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Problems in physiological experimental animal models investigated with factorial design

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Abstract

In the present study we investigated four variables using factorial design to decide if any of these could explain the variations in the control measurements of interstitial fluid pressure (P_{if}) in rat trachea that were experienced. This approach requires only a fraction of the animals normally needed when studying each factor separately. P_{if} in tracheal tissue was measured with the servocontrolled counterpressure system using sharpened micropipettes. The measurements were performed over a period of 60 min and are presented as mean for every 15 min period. The factors investigated in the study were: three strains of female rats (Strain) two brands of diets (Food); two breeder companies (Source); and finally two batches of the same set of animals to repeat the experiment twice (Week), using a total of 48 animals. There was a highly significant effect within Strain the first week ($p = 0.007$), but this response was not observed the second week. The interaction between Strain × Week was significant ($p = 0.007$) while the main effects Strain or Week alone were not significant. The response pattern for Strain and Food was inconsistent for the two experimental weeks studied. These experiments made it possible for us to simultaneously test several factors and exclude these factors as the reason for the observed changes in our experiments since the experiments did not allow the conclusion that one or several of these factors could explain the variation in P_{if}.

Keywords: Factorial design; Animal model; Wistar; Fisher F344; Brown Norwegian; Diet

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Introduction

The standardization of animal experiments in physiological research is important to increase reproducibility at different times and locations with similar results. In animal experiments the rules of the 3 R's: Reduction, Refinement and Replacement (Russell and Burch, 1959), should be implemented in planning and performing experiments (Flecknell, 2002). Much focus has been put on standardizing environmental factors known to cause unintended variations of the experiments. Some of the parameters that are known to affect results are temperature, air changes, air humidity, light–dark cycles, microbiological conditions (Poole et al., 1987). The current investigation, demonstrate how such a study can be performed, when it becomes necessary to perform experiments involving several variables and groups.

The experiments were performed since interstitial fluid pressure (P_{if}) in rat trachea in control animals was observed to be lowered, a response otherwise seen in acute inflammatory reactions such as following mast cell degranulation and neurogenic inflammation (Koller and Reed, 1992; Koller et al., 1993; Woie et al., 1993; Woie and Reed, 1994). These findings occurred irregularly, but some observations could suggest that the differences could be due to variability in animal stocks or to conditions in the animal facility.

The transcapillary fluid filtration is created by the imbalance between the osmotic and the hydrostatic pressures over the microcirculatory wall, giving a net filtration pressure of 0.5–1 mmHg in peripheral tissues as skin and skeletal muscle (Aukland and Reed, 1993; Wiig et al., 1981). The lowering of P_{if} will increase the transmural driving pressure during the initial phase of an inflammatory challenge. The lowering of P_{if} measured in tracheal tissue of rats following an inflammatory challenge ranges from -4 to -10 mmHg (Gjerde et al., 1997, 1998, 2000; Koller and Reed, 1992; Wei et al., 1998; Woie et al., 1993; Woie and Reed, 1997; Woie and Reed, 1994; Woie and Reed, 1996a; Woie et al., 1996). Under normal (non-inflammatory) conditions P_{if} is slightly subatmospheric and the mean average and standard deviation measured over 60 or 90 min registration period for trachea is -1.45 ± 0.36 mmHg. This average is taken from a total of 13 studies and 17 series of experiments containing all together 132 control animals giving a average of 7–8 animals per series (Gjerde et al., 1997, 1998, 2000, 2002, 2003; Koller and Reed, 1992; Koller et al., 1993; Wei et al., 1998; Woie et al., 1993, 1996; Woie and Reed, 1994, 1996, 1997). Figures from previous studies (Koller et al., 1993; Gjerde et al., 2002) illustrate that the levels of P_{if} in vehicle treated animals are constant throughout the 60 min recording period. The slightly negative P_{if} is one of the forces controlling the filtration over the microcirculatory wall into the interstitium and also acts as a filling pressure for the initial lymphatics (Aukland and Reed, 1993; Levick, 1991). For all previous studies on changes in P_{if} in rat trachea, female Wistar rats were used, mainly provided from M&B AS (P.O Box 39, DK-8680, Ry, Denmark) and with a very few exceptions delivered by Harlan UK Ltd (Shaw's Farm, Blackthorn, Bicester, Oxon, OX6 0TP, England). These animals were, over the years, first fed with SDS (Special Diets Services, UK), RM1 Maintenance Diet and later, due to changes in the diet routine in the animal facility, the animals were fed with B&K (B&K universal UK)

Rodent Low Protein Diet. Over a period of several months we began to observe variable values and quality in the control measurements of P_{if} with values considerably more negative, i.e. with values similar to those observed in inflammation.

Environmental factors were suspected to cause the problems since even minor changes in the airway histology are common findings in laboratory animals and often seen in rats without any serological evidence of viral infection (Greaves and Faccini, 1984), although they are usually not considered to be of pathological importance (Greaves and Faccini, 1984). Several environmental factors were improved such as reducing stress by taking special care in the animal handling before and during the experiments. We also improved the conditions at the animal facility by isolating the animals in separate rooms or by placing them in a ventilated cupboard as well as changing the time of the acclimatization period after transport. The conditions with normal P_{if}, i.e. the control measurements of P_{if} were only partially restored, but with little consistency and with no clear preferences towards any environmental factor. McDonald and collaborators (McDonald, 1988, 1992) have demonstrated that animals infected with *Mycoplasma pulmonis* had increased response to neurogenic inflammation. Our animals were delivered by a supplier with regular health monitoring programs and tested negative for mycoplasma as did the sentinels from our animal facility during the actual time period. Also animals were kept in isolators to avoid infection after arrival to the animal facility.

The aim of this study was to investigate if genetic or environmental factors were able to explain the observed variability in P_{if}. Also, this report demonstrates the use of factorial design to proceed systematically in the study of multiple factors by avoiding the use of large amount of animals. The conclusion of the study is, however, that none of the factors investigated alone or together explained the observed changes in P_{if}.

Material and methods

Female rats from two inbred strains, Fisher F344 (F344/NMol and F344/NHsd) and Brown Norway (BN/Mol and BN/SsNOlaHsd) as well as one outbred stock Wistar (Mol:WIST Han and HsdOla:WI) were supplied in equal numbers from both M&B AS (P.O Box 39, DK-8680, Ry, Denmark) and Harlan UK Ltd (Shaw's Farm, Blackthorn, Bicester, Oxon, OX60TP, England). The animals from both Week groups were acclimatized for 3 weeks at the following conditions before the experiment: Relative humidity (RH) 50–55%, temperature range of 19–21°C, and 12 h light cycle and were fed either SDS (Special Diets Services, UK), RM1 Maintenance Diet or B&K (B&K universal UK) Rodent Low Protein Diet and tap water ad libitum. Both diets have similar protein source i.e. extract of soya bean meal and whey powder. The respective unit percentage of the nutrients in the two different diets, for crude protein was 14.70% and 14.59%, respectively, and for digestible crude protein 13.30% vs 12.33%, respectively.

Table 1. Overview of the experimental design

Strain	Animal supplier	Diet		Total # animals
		SDS Maintenance	B&K Low protein	
Wistar	M&B, DK	2	2	4
	Harland, UK	2	2	4
F344	M&B, DK	2	2	4
	Harland, UK	2	2	4
Brown Norway	M&B, DK	2	2	4
	Harland, UK	2	2	4
Total		12	12	24
The experiments were repeated twice			Total	48

A total of 48 rats were used and divided into two experimental weeks each with 24 rats. The two groups of animals forming the Week-groups, differed in arrival time by approximately 2 weeks and had an acclimatizing time of 3 weeks before the experiments took place. Three weeks of acclimatization has previously been observed as more than enough time to induce changes in P_{if}. The animals were of approximately the same age (around 6 weeks, but varied in weight at the time of the experiments from 150 to 250 g. This variation is due to the fact that inbred strains are often smaller in size than outbred at the same age. The animals were kept isolated from other experimental animals, in separate rooms provided with four racks to separate the two food groups. Each rack contained 12 rats from the three different strains and each cage contained only two animals. An overview of the experimental design is presented in Table 1. Although, we were not able to measure P_{if} in two of the 48 rats (i.e. one Mol:WIST Han; B&K; Week1 and one F344/NHsd; B&K; Week2), the statistical analysis was not impaired by a reduction to 46 animals. The procedures were carried out with the approval of and in accordance with the regulations of the National Animal Research Authority.

Experimental protocol

The rats were anaesthetized with sodium pentobarbital (initial dose of 50 mg/kg i.p. and supplementary doses when needed). A branch of the femoral vein was cannulated for injection of potassium chloride (0.5–1 ml) to induce cardiac arrest. The subsequent measurements of interstitial fluid pressure in the tracheal tissue lasted for a total of 60 min, with measurements reported for four time periods of 15 min each.

Measurements

Interstitial fluid pressure (P_{if})

After induction of circulatory arrest an extra-thoracic portion of trachea was exposed and the muscle overlying the trachea was split longitudinally. The tracheal surface was covered with mineral oil to avoid desiccation. The time required for the experimental preparation after cardiac arrest was 1–2 min. Measurements of P_{if} were initiated soon thereafter on the abluminal portion of trachea and between the cartilage rings using sharpened glass capillaries (4–10 μm) connected to a servocontrolled counterpressure system (Wiederhielm et al., 1964; Wiig et al., 1981) and continued until 60 min after circulatory arrest. The glass pipettes were filled with 0.5 M NaCl colored with Evans blue. Micropuncture was performed under visual guidance with a microscope (Wild M3C, Heerbrugg, Switzerland) and care was taken to minimize stretch or compression at the site of puncture (Wiig et al., 1981). The measurements were accepted when the following criteria were met: (1) No change in recorded pressure when increasing feedback gain; (2) Suction applied by the servocontrolled pump should increase electrical resistance in the pipette, verifying open communication to interstitial fluid because of the lower tonicity of the fluid entering the pipette; (3) The baseline measurements before and after P_{if} registration were unchanged. The latter measurement was performed in a plastic cup filled with saline placed at the level of the site of puncture. All surgical manipulations on or near the trachea were performed after circulatory arrest to avoid local inflammation that may increase interstitial fluid volume and confound accurate estimates of P_{if} .

Statistical methods

A factorial design was used, with all cross-classifications present. The statistical model was the following repeated measures ANOVA, using JMP (1) from SAS Institute (SAS Institute, 1993):

$$(Y1 - Y4)_{ijkl} = \mathrm{MEAN}_{ijkl} + \mathrm{FOOD}_i + \mathrm{SOURCE}_j + \mathrm{STRAIN}_k + \mathrm{WEEK}_l + e_{ijkl}, \quad (1)$$

where $Y1 - Y4$ were the consecutive recordings of P_{if} at 15 min intervals within an hour, FOOD were of two brands ($i = 2$), SOURCE were two commercial breeders ($j = 2$), STRAIN represented the two inbred strains and one single outbred ($k = 3$), WEEK are the two consecutive weeks of repeated experiments ($l = 2$) and e_{ijkl} is the random error. The normality test was performed and passed prior to performing the main analysis. The analysis started with a full factorial resolution, then we only kept the significant interaction terms plus the main effects in the final model. The number of animals needed for the experiment was estimated according to Mead's resource equation (Mead, 1988). We included sufficient animals to provide information on all two-way interactions, i.e. $n = 48$. Results are presented as the least-squares means from (1), LS Mean $\pm$ SEM

Results

The effect of rat strain was highly significant in the first week of the experiment (Table 2) and the different rat strains had different response profiles on P_{if} (Fig. 1, upper left). However, this effect did not appear on other rats the following week (Fig. 1, upper right). There was a significant effect of the interaction term Strain × Week, while none of the main effects, Strain or Week were significant although Strain was of borderline significance (Table 2). This implies that the response pattern for each rat strain was different in the two experimental periods. There was a significant effect of time on the repeated measurements, i.e. the curves were not horizontal. The P_{if} was falling throughout the 1 h recording procedure a pattern recognized in an inflammatory response (Koller et al., 1993).

There was a significant interaction term for Time × Strain in the experiment done in the first week, indicating that the reaction curves for the different strains had different shapes and/or slopes. However, the following week's experiment produced response curves that were not different between strains. The strains contribute differently to this effect. Wistar and F344 are the main contributors to the difference between the weeks in the three last time periods, as can be exemplified in a Strain × Week leverage plot for the 16–30 min time period (Fig. 2). There was a significant interaction term for Strain × Food (Table 2). This effect could be seen in Fig. 1, where the mean response curves for the feeding groups appear in the reverse order in the consecutive weeks. Only 12 of the 46 rats behaved like 'normal controls' (i.e. with horizontal curves throughout the 1 h recording and no mean total P_{if} less than −2 mmHg, (Koller et al., 1993). A log-linear analysis could not identify an increased likelihood of 'normal controls' in any experimental group (data not shown). When the statistical analysis was performed with $n = 23$, i.e. in effect by stating that only one measurement can be obtained from all animals in the same cage, the statistical differences above disappeared (see below).

Table 2. *F*-values and significance levels from the four time periods

Effect	Both weeks		Week 1		Week 2	
	Exact *F*	*p*-value	Exact F	*p*-value	Exact *F*	*p*-value
All between	2.93	0.016	4.66	0.013	1.01	0.43
Food	0.12	0.72	3.02	0.1	1.75	0.21
Source	0.58	0.45	0.96	0.34	0.004	0.95
Strain	2.87	0.073	7.33	0.007	1.26	0.31
Week	0.02	0.89	—	—	—	—
Food × Week	4.73	0.038	—	—	—	—
Strain × Week	5.93	0.007	—	—	—	—
Time	15.09	<0.0001	16.63	0.0001	3.61	0.049
Time × Strain	0.96	0.45	2.77	0.003	0.64	0.69

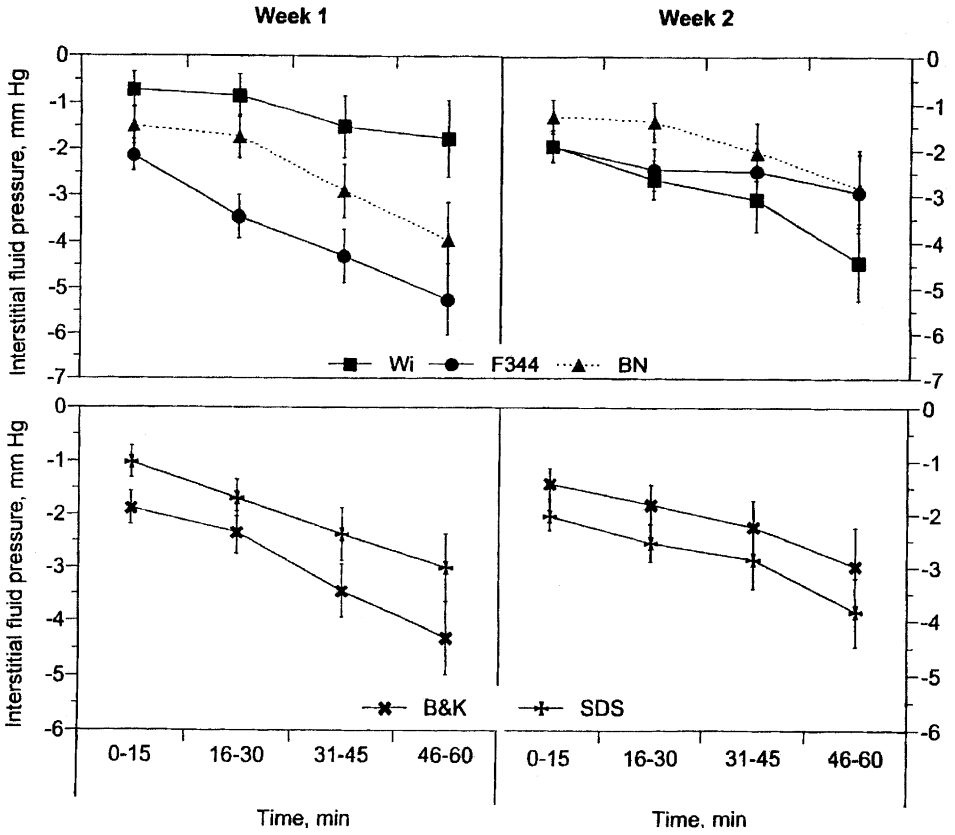

Fig. 1. LS Mean P_{if} values for the rat strains/stock at the four sampling intervals (top) and the groups fed the two different food brands (bottom). The graphs show the values from the first experiment (left) and the repeated experiment (right). Error bars indicate $\pm$SEM.

Discussion

The purpose for using the factorial design was to increase the amount of information and the generality of the results of an experiment (Festing et al., 2002). It is a powerful alternative for doing several smaller experiments for each factor and also allows testing for interactions between these factors (Festing et al., 2002). Factorial designs are used to investigate whether a response to one treatment is the same across the levels of all the studied variables (Festing et al., 2002).

The observation that when the measurements from each cage are averaged, the statistical difference disappears, may have two explanations and implications. First, it does in fact strengthen our conclusion that we cannot in this study pinpoint a single causative factor for the observations that were the starting point for this study. Also, since the factors reaching significance in Weeks 1 and 2 were different, this

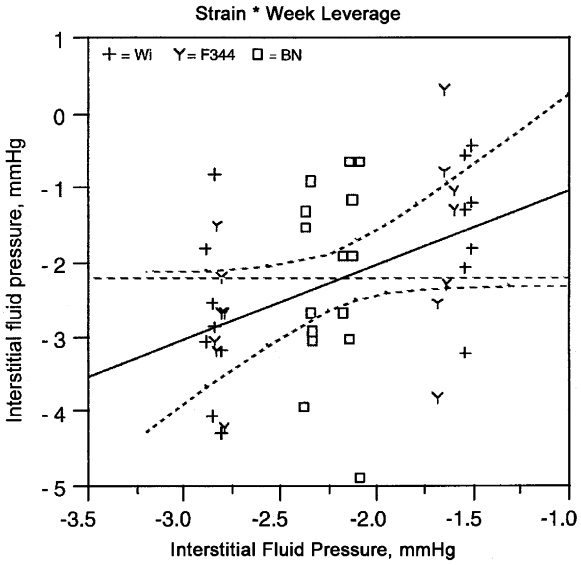

Fig. 2. Leverage plot for the 16–30 time period of the interaction term for Strain × Week. This significant effect ($p < 0.05$) is caused by the values (rats) far away from the center of the plot. BN rats contribute less than Wistar and F344.

would seem to be an argument in the same direction, i.e. that the statistical significances were borderline and could not be verified, either when studied in the subsequent week, or when reducing the power of the statistical analysis.

However, if the experiments were to be performed according to the procedure outlined above by averaging all corresponding measurements from one cage, and perform the subsequent statistical analysis on such average data, rather than on data from one single experimental animal, this is in fact testing a different hypothesis. However, depending on the hypotheses and questions asked, both approaches seem to be valid. In the experimental situation leading to the present study, there was seemingly a random appearance of animals that behaved like "experimental" animals while they were in fact controls. In the measurements performed in this study, these animals appeared randomly throughout the cages. The effect of averaging the data from one cage is therefore to minimize an effect that should be present, simply due to the averaging. Therefore, it is not surprising that after averaging the statistical significances disappear.

Furthermore, the averaging procedure has another principal discussion attached to it, namely whether the number of experiments actually performed should be the total number of registrations in an animal, the average of the measurements *within* one animal or the average *between* different animals in the same cage. In accordance with accepted practice for physiological experiments, we have averaged the measurements *within* one animal and used this for the subsequent statistical analysis. Our argument in favor of this choice of procedure is that in all previous similar

studies, among them the ones leading to the present one, the averaging and analysis have been outlined as described above. We feel that if the study was to be performed based on averaging the animals within one cage, the proper design would actually be to have one animal per cage. However, this will have such implication on animal care and cost, that it would seriously limit the number of experiments based on cost.

Diet

The rationale for testing the two types of diet chosen here, was based on the fact that there had been performed changes in the diet routine in the animal facility and due to other research groups necessity to develop old specimens of rats. A diet from another supplier, with lower protein, starches and sugar content, was put to use to avoid excessive growth in these animals. It was then decided that all animals in this facility should be fed with the same low protein diet.

Animals used in our experiments were relatively young and were only housed in the animal facility for a short acclimatization period before the experiment. Another argument for testing the response of P_{if} on diet is the fact that the source of fatty acid can modulate the immune response. Likewise, arachidonic acid metabolites, derived from fatty acids, are important in the inflammation cascade and may modulate the inflammatory response (Demarne et al., 1979; Hoverstad and Midtvedt, 1986). Also a change of gut microbiological flora can affect the metabolism of fatty acid (Bruckner and Gannoe-Hale, 1989).

The significant effect of the interaction between Food and Week could suggest a variation in the food to be of importance. The inconsistency between the two weeks could indicate that one of the food brands did not have the same composition within the same batch, which is occasionally observed (Baumans et al., 1993).

Supply of animals, source

It is well known that stress like shipping, can provoke latent disease caused by opportunistic microbes as with shipping fever caused by *Pasturella* spp. in cattle or Glasser's disease caused by *Hemophilus* spp. in pigs (Blood and Radostits, 1989). Immunosuppression caused by increased endogenous cortisol due to stress is the usual explained mechanism behind this (Blood and Radostits, 1989).

The animals were supplied by breeders with a regular health monitoring program, and did not carry pathogens according to the FELASA guidelines. There is still a possibility that the animals were infected after they arrived to our facility and this was tested by performing experiments with animals housed in isolators. However, this gave a distribution of P_{if} similar to what was experienced in the "problem period" (unpublished data). The use of isolators was therefore not included in the factorial study. Even if the animals are tested and specific pathogen free (SPF) for defined pathogens, this gives limited information and only tells which microbes that the animals do not have. The animal's normal bacterial flora is seldom characterized or reported. Complete control of the bacterial and microbiological flora is obtained in germ free animals delivered by cesarean section, be colonized with a defined

microbiological flora, and housed isolated for the rest of their life (Heidt et al., 1990). Such animals were not used in this study.

McDonald (McDonald, 1988, 1992) demonstrated that *M. pulmonis*, Sendai virus and coronavirus cause an increased susceptibility to neurogenic inflammation in the rat trachea, which is an inflammatory reaction that also induces lowering of P_{if} (Woie et al., 1993). The current inability to achieve 'normal control' measurements of P_{if} could therefore result from an infection from some of the agents above. However, neither mycoplasma, Sendai virus nor coronavirus have ever been detected in sentinel animals during this period.

Rat strain

An inbred strain is obtained by having brother and sister mating from the same branch for at least 20 generations. They are homozygous at more than 99% of the loci (Festing, 1979; Poole et al., 1987). The genetic status of an inbred strain will stay constant over time, like an immortal clone of identical individuals and changes only occur by mutations. An outbred stock like the Wistar rat, is obtained by breeding a closed colony of genetically undefined animals for at least 4 generations. The genetics of an outbred stock might change over time due to genetic drift as a result of random mating within the population. The use of inbred strains gives a larger statistic precision by use of fewer animals. However, they are more sensitive to environmental influences than outbred stocks. The overall choice was to use several different inbred strains. This will increase the generality of the experiment and still have the same genetic material over time. The rationale for using the outbred Wistar is that it has been used for a long time in similar experiments.

The different strains behaved differently in the two experimental weeks. Although inbred animals are more sensitive than outbred to changes in the environment (Festing, 1979) the combined effect of strain in the total experiment is not significant since the two repeated experiments gave opposite ranking of the strain mean values. The Strain × Week interaction is actually a study of a genotype–environment interaction. The tendency in these experiment is that BN rats show the highest degree of consistency in the experiments between Week, while the outbred Wistar rats give slightly less consistent results than F344, i.e. BN $\gg$ F344 > Wistar. This may suggest that the variation in the response in Wistar rats was due to the genetic heterogeneity of this stock, since two batches of Wistar rats are genetically different.

The differences between the strains could be due to variation at a receptor expression level. Pauwels and collaborators (Germonpre et al., 1995; Joos et al., 1994; Pauwels et al., 1995) report different susceptibility to plasma protein extravasation following neurogenic inflammation in trachea of different rat strains. They identified several genetic differences between the F344-rat and BDE-rats, among others in the mechanisms involved in the plasma protein extravasation in the airways following substance P or capsaicin, since these agents released 5-hydroxytryptamine from the mast cells in F344-rats but no BDE-rats (Pauwels et al., 1995).

This study has shown that even though we find differences in P_{if} measurements with regard to several of the parameter investigated, none of them were able to restore 'normal control' P_{if}, i.e. not less than -2 mmHg. Although the experiments were inconclusive with regard to which factor that by itself or in combination with others caused the variability in P_{if} the study demonstrates how several factors can be investigated using a minimum of experimental animals.

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