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The Effects of Drugs on Mice of Three Strains Using 3^2 Factorial Designs

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ABSTRACT

This paper examines the effect of drugs on mice of three strains using 3^2 factorial designs. The weight, Red Blood Cell (RBC) and the White Blood Cell (WBC) responses of three strains of mice when they are dosed with control, chloramphenicol at low level (500mg/kg) and high level (2500mg/kg) was analyzed using 3^2 factorial designs. The analysis of the RBC shows that there is no interaction effect between the strains of mice and the three dose level. The three strains of mice and dose level are found not to be significantly different from each other. For the WBC, there is a significant difference among the three dose level. There is no interaction effect between the strains of mice and the dose level and the three strains of mice are not significantly different from each other. The analysis on weight gain of three strains of mice dosed with control, low level (450mg/kg) and high level (2400mg/kg) of chloramphenicol shows that the main effect (A), dose level are not different significantly while the main effect (B), strains of mice are different significantly. There is also no interaction effect between the three dose level and strains of mice at 5% level of significance.

Keywords: Factorial Design, Mice Strains, Effect of Drugs, Red Blood Cell

INTRODUCTION

There is considerable scope for reducing the number of animal usage and scientific resources in research by designing better experiments. Some experiments are performed repeatedly with only minor variations and even very small improvements in the design can lead to substantial savings on animals' usage over a period of time. Animal experiments form a necessary part of the late stages of the drug discovery process. An animal model may be used to screen large numbers of compounds with only the identity of the compounds changing between experiments. A typical experiment, which may involve three or four groups of approximately eight animals treated with different candidate compounds and a largely control group, may have the aim of findings the compound that have potentially useful effects. Batch of vaccines and other biological are often tested in animals using a standard protocol, with the aim of measuring the biological activity or toxicity of the batch. Even in base research, some procedures (e.g. the preparation of DNA) use complex methods that may be used repeatedly even tough individual experiment may vary. If all of these experiments and associated techniques were optimized to use the smallest number of animals consistent with detecting a given response, there would be a substantial reduction in animals use and important savings in scientific resources.

One method of optimizing and showing the effect of certain drugs of experiments is to use factorial experiment. The challenge is usually what level of these factors would lead to an experiment with the greatest sensitivity. The aim is usually to maximize count ratio so that the numbers of experimental subjects required to detect a given treatment response is minimized by using power and sample size calculations. The procedure involves using a control and a known positive control treatment and attempting to maximize the mean difference. In this research work, variables investigated for their influence on the treatment effect are termed "factor". It is also useful to know which of these factors are relatively

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unimportant in influencing response so that less attention is given to controlling them. The factors to be studied can be any variables the investigator an control including direct animal – related characteristics (e.g. sex, strain, age and dietary and health status) and aspects of the environment (e.g. cage, group size, bedding material, and environmental complexity). There are also many protocol–specific factors (e.g. methods of preparing the animals models: dose level: timing, route and method of administration of test compounds and methods and timing of observations).

When complex protocols are involved in making the final observations (e.g., in the preparation and hybridization of DNA in micro assay experiment may also need to be investigated. Sometimes animals are used as sources of tissues or cells in vitro experiments and the factors that influence the outcome of these experiments can affect the numbers of animals that are needed. Often this type of experiment will initially involve 2^n or 3^n factorial where **n** factors are studied, each set at three or two levels.

This research examines the effect of drugs on mice of different strains by employing a 3^2 factorial design (i.e. 2 factors at 3 levels each). The use of factorial experimental design is to determine which factors influence the outcome of the experiments and the optimum levels of those factors so that future experiments can be designed to have the greatest possible sensitivity. The use of factorial design is an integral part of the development of a good animal model. The aim of the research is to know if there is any significant difference in WBC counts of strains, to know if there is any significant difference in dose level, and to know if the strains differ in their response to chloramphenicol (the interaction). The experimenter administered a control treatment; chloramphenicol 500mg/kg and 2500mg/kg to three different strains of mice with 2 replicates for 3days. The drugs (factor A) is at three level i.e. control, low dose (500mg/kg) and (2500mg/kg) while the mice (factor B) is also at three levels. A total of 27 mice were used for the experiment where nine were selected separately to measure the White Blood Cell counts. The chloramphenicol administered was simulated by subtracting 50mg/kg from low dose level and 100mg/kg from high dose level.

REVIEW OF RELATED LITERATURE

[1] compared the behavioral effects of drugs between two strains of mice. The BALB/C and CBA mice were injected with Caffeine (3, 10, 30, 100mg/kg), diazepam (1, 3, 10mg/kg) and chloral hydrate (10, 30, 100mg/kg) respectively. Ten minutes after injection, the locomotors activity in the open field was recorded for two hours. The total distance ratio, the distance ratio to total distance and the time in central region were analyzed for each drugs. Thirty minutes after injection, the latent time in the passive avoidance test was measured in a shuttle box. The researchers reported that caffeine and diazepam prolonged the latent time, and ephedrine and chloral hydrate decrease the latent time, but there were no differences between the two strains. The two strains of mice exhibited significant differences in the total distance after injection of ephedrine 10mg/kg, diazepam 3mg/kg and chloral hydrate 100mg/kg. Compare to CBA mice, BALB/C mice exhibited an increase in the distance ratio, and time in central region after injection of ephedrine 10-100mg/kg, but a decrease after injection of diazepam 3-10mg/kg.

Much toxicological research continues to be done using genetically undefined “outbred” stocks of mice’s stocks has been made repeatedly in the literature over a period of more than two decades. Also, very few studies are conducted using more than one strain, with the result that genetic variation in response is seldom apparent to the investigator, [2].

[2] reported qualitative and quantitative strain difference in the hematological response to chloramphenicol succinate C (CAPS) when administered by gavage at 500-2500mg/kg for 7days, to four inbred strains of mice (C3H/HE, CBA/Ca, BALB/C and

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C57BL/6) and one outbred stock (CD-I). CAPS caused anaemia and reticulocytopenia in all mice strains but not in the outbred CD-I stock.

Further analysis by [3] reported that all four inbred strain showed significant ($P < 0.01$) responses to CAPS at lower dose levels than in CD-1 mice, which were phenotypically more variable than the inbred strains (animals). A simulated experiment using a sample records from the present study showed that the use of two mice at each dose level using CD-1, CBA,BALB/C and C57BL/6 C 48 total mice) would have given a more sensitive experiment than the use of 47 CD-1 mice alone. It also shows that the response is partly strain dependent. These studies provide additional evidence that inbred strains, because of their great sensitivity and other valuable properties should be more widely used in toxicology.

Chloramphenicol has been widely used in the treatment of serious infection including typhoid, fever and meningitis. However, the drug is Haematoxicity in man including firstly, a reversible dose-dependent anaemia which develop during treatment. Secondly, an often fatal aplastic anaemia with pancytopenia and cellular marrow and thirdly, leukaemia [4].

[5] investigated the piaemotoxicity of chloramphenicol succinate (CAPS) in female CD-1 mice in repeat dose studies, to compare the response with the reversible anaemia reported in man. Studies in male BALB/C were also carried out. CAPS were gavage daily to mice at dose level from 800-2000mg/kg for seven days. Values were significantly reduced for reficulocytes at 1700 and 2000 mg/kg, and for erythrocytes (RBC), Haematoxicity (HCT), and haemoglobin (H6) at 2000mg/kg platelet and White Blood Cells (WBC) counts were unaffected. The researchers dosed strains mice with CAPS at 1400mg/kg for 10days and sample at 1, 4 and 15 days after the least dose. At day 1 post dosing, RBC, HCT and H6 values were significantly reduced, but returned to normal (or above normal) by day4 0r 15. CAPS from 2000-4000mg/kg were gavage to rats daily for 19days. Hb values were significantly lower at 3600and 4000mg/kg, reficulocytes were not reduced. WBC and platelet counts, in general were unaffected. [5] induced heamatological changes showing close parallels with the chloramphenicol-induced reversible anaemia seen in man.

RESEARCH METHODOLGY

The 3^k Factorial Design

The 3^K factorial design is a further development on the 2^K factorial design. The 3^K factorial design is a k-factor design where each factor is of three levels. The levels of each factor are referred to as the low, intermediate, and high levels respectively. The low, intermediate and high levels will respectively be represented symbolically by the digits 0, 1, and 2. Thus, every treatment combination in the 3^K experiment will be designated by k-digits of the combinations of 0, 1 and 2. For example, in a 3^2 factorial design with factors A and B, 00 implies the treatment combination of low level of A and low level of B; 01 is the treatment combination of low level of A and intermediate level of B, while 12 is intermediate level of A and high level of B treatment combination. So it is for other treatment combinations.

The 3^2 Design

The 3^2 design is the simplest of the 3^k designs. It is a 2-factors design with each at three levels. Given the factors as A and B, there are three levels of A : 0, 1, and 2 and three levels of B; 0, 1, and 2. The treatment combinations are 00, 01, 02, 10, 11, 12, 20, 21, and 22. There are total of nine treatment combination which is obtainable from $3^2 = 9$. Therefore the total degree of freedom between the treatment combinations for a one – replicate experiment is 8. The main effect A and B have two degrees of freedom each the interaction AB has four degrees of freedom. In r replicates of a 3^2 experiment, the total degree of freedom is $3^2r - 1$. The error degree of freedom is given by:

df (error) = df (total) – df(A) – df(B) – df(AB).

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$= 3^2r - 1 - 2 - 2 - 4 = 3^2r - 9 = 3^2 (r - 1)$.

The sum of squares of the main effects and interactions could be computed using Yates' algorithms because of the exponential increase in the number of treatment combinations as k increases.

Linear and Quadratic Components

The factors whose effects are investigated in design and analysis of experiments could either be quantitative or qualitative factors. Quantitative factors are those factors whose levels could be represented on the number line while qualitative factors are those factors that are distinguished possibly only by their nature. Three points are considered in the 3^k design with each level of factor as a point with a qualitative factor. Given any three points, only a straight line or curve could pass through those three points. This is pointing to the fact the three levels of a factor in the 3^k design can either be collinear, that is, they lie on the same straight line, or they are curvilinear, that is, they lie on the same curve. Since only three points are considered, the curve can only be a parabola. Therefore, the three levels of factor can be modeled by either a linear equation or a quadratic equation. This view gave rise to the linear and quadratic components of an effect in the 3^K design.

Computation of Sum of Squares

The Sum of Squares of effects can be computed in the 3^K design using the usual method for factorial design. However, the Yates' algorithm has also been developed for the 3^K factorial design.

Yates' Algorithm for the 3^2 Design

The procedure for computing the sum of squares using Yates' algorithm is as follows: The treatment combinations are written down in the standard order in the treatment combination column of the ANOVA table. For the 3^2 design.

TABLE 1: Yates' algorithm for 3^2 designs.

Treatment combinations	observation	(1)	(2)	Effect	Divisor	Sum of Squares
00						
10				A_L	2^F3^tr	$\frac{(column)^2}{2^f3^tr}$
20				A_Q		
01				B_L		
11				$AB_{L\times L}$		
21				$AB_{Q\times L}$		
02				B_Q		
12				$AB_{L\times Q}$		
22				$AB_{Q\times Q}$		

The procedure commences from column (1) as follows: The first three observations in column (1) consists of sums of each set of three observations in the observation column.

The next three entries are obtained by subtracting the first observation in each of the sets of three from the third. The last stages for entries in column (1) is obtained by subtracting twice the middle observation in each set of three and subtract the result from the sum of the first and the third observations. Thus, the entries in column (1) are computed. The procedure is repeated for entries in column (1) under column (2). The computation is completed in column (2) for the 3^2 design.

The divisor corresponding to each entry in column (2) is obtained from the expression $2^f \times 3^t \times 2$ where "F" is the number of factors in the effect considered, "t" is the number of factors in the experiment minus the number of linear terms in the effect and "r" is the number

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of replications, r=2. The last column is the column for the Sum of Squares is obtained by squaring the entries in column (2) and divide by the corresponding divisor.

$SS_E = \frac{(column)^2}{divisor}$

RESULTS AND DISCUSSION

TABLE 2: Summary of data collected on WBC of mice of three Strains dose with a control, chloramphenicol at 500mg/kg (low level) and 2500mg/kg (high level).

Dose Level			
Strains of mice	Control	Low Level (500mg/kg)	High Level (2500mg/kg)
C3H	5.23, 3.65	4.10, 4.23	7.88, 7.62
CBA	4.03, 5.88	1.51, 3.61	5.43, 5.42
CD-1	4.67, 4.60	3.12, 3.01	5.57,6.23

Analysis using 3^2 Factorial Designs for WBC response

The treatment combinations are written down in the standard order in the treatment combination column of the ANOVA table.

For the above table of data collected, the control is designated as "0", the low level (500mg/kg) is designated as "1" and the high level (2500mg/kg) is designated as "2" for the dose level.

Similarly for the strains of mice, C3H is designated as "0", CBA as "1" and CD-1 as "2".

Dose level is factor A and a strain of mice is factor B.

Main effect A is dose level

Main effect B is strains of mice

Interaction effect AB is dose level × strains of mice.

For the 3^2 designs, the standard orders of the treatment combination are: **00, 10, 20, 01, 11, 21, 02, 12, and 22 (9 treatment combinations).**

TABLE 3: Table showing various levels of factor A and factor B and their values.

STRAINS OF MICE (B)	DOSE LEVEL (A)		
	0	1	2
0	8.88	8.33	15.5
1	9.91	5.12	10.85
2	9.27	6.13	11.8

Grand total $Y_{...} = 85.79$, number of treatment t is 3, number of block is 3, and replicates is 2, N is the total number of observation = r×t×b = 2×3×3 = 18

Correction factor C.F = $\frac{Y_{...}^2}{N} = \frac{85.79^2}{18} = 408.885$

TABLE 4: Yates' algorithm for 3^2 Factorial Designs.

Treatment combination	Observation	(1)	(2)	Effect	Divisor	Sum of squares
00	8.88	32.71	85.79			
10	8.33	25.88	10.09	A_L	$2^1\times3^1\times2$	8.48
20	15.50	27.2	27.05	A_Q	$2^1\times3^2\times2$	20.33
01	9.91	6.62	-5.51	B_L	$2^1\times3^1\times2$	2.53
11	5.12	0.94	-4.09	$AB_{L\times L}$	$2^2\times3^0\times2$	2.09

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21	10.85	2.53	1.09	$AB_{Q\times L}$	$2^2\times3^1\times2$	0.050
02	9.27	7.72	8.15	B_Q	$2^1\times3^2\times2$	1.85
12	6.13	10.52	7.27	$AB_{L\times Q}$	$2^2\times3^1\times2$	2.20
22	11.8	8.81	-4.51	$AB_{Q\times Q}$	$2^2\times3^2\times2$	0.28

The divisor corresponding to each entry in column (2) is obtained from the expression $2^f\times3^t\times2$ where "F" is the number of factors in the effect considered, "t" is the number of factors in the experiment minus the number of linear terms in the effect and "r" is the number of replications, r=2.

Calculation for the Sum of Squares

Sum of squares, SS = $\frac{(column)^2}{divisor}$

$SS_{A_L} = \frac{(10.09)^2}{12} = 8.48$ $\quad SS_{A_Q} = \frac{(27.05)^2}{36} = 20.33$

$SS_{B_L} = \frac{(-5.51)^2}{12} = 2.53$ $\quad SS_{AB_{L\times L}} = \frac{(-4.09)^2}{8} = 2.09$

$SS_{AB_{Q\times L}} = \frac{(1.09)^2}{24} = 0.050$ $\quad SS_{B_Q} = \frac{(8.15)^2}{36} = 1.85$

$SS_{AB_{L\times Q}} = \frac{(7.27)^2}{24} = 2.20$ $\quad SS_{AB_{Q\times Q}} = \frac{(-4.51)^2}{72} = 0.28$

Sum of Squares Total, $SS_T = \sum\sum\sum Y_{ijk}^2 - C.F$

$$SS_T = \left(5.23^2 + 3.65^2 + 4.23^2 + \ldots + 5.57^2 + 6.23^2\right) - \frac{85.79^2}{18}$$

$SS_T = 452.1271 - 408.885 = 43.24$

Sum of Square Error, $SS_E = SS_T - SS_A - SS_B - SS_{AB} = 43.24 - 28.81 - 4.38 - 4.62 = 5.43$

TABLE 5: Analysis of variance table

Source of variation	***Degree of freedom***	***Sum of squares***	***Mean sum of squares***	F_O
A	2	28.81	14.405	23.89
A_L	1	(8.48)		
A_Q	1	(20.33)		
B	2	4.38	2.19	3.63
B_L	1	(2.53)		
B_Q	1	(1.85)		
AB	4	4.62	1.155	1.92
AB_{LL}	1	(2.09)		
AB_{QL}	1	(0.05)		
AB_{LQ}	1	(2.20)		
AB_{QQ}	1	(0.28)		
Error	9	5.43	0.603	
Total	17	43.24		

Hypothesis Statement

Main effect A, dose level

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H_0: There is no significant difference among the main effect A i.e. among the dose level.
H_1: There is a significant difference among the main effect A i.e. among the dose level.

Main effect B, strains of mice
H_0: There is no significant difference among the main effect B i.e. among the strain of mice.
H_1: There is a significant difference among the main effect B i.e. among the strain of mice.

Interaction effect AB, dose level × strains of mice
H_O: There is no interaction effect between dose level (A) and strains of mice (B).
H_1: There is interaction effect between dose level (A) and strains of mice (B).

Test Statistic
F-ratio from the ANOVA table:
F-ratio for dose level = 23.89
F-ratio for strains of mice = 3.63
F-ratio for interaction between dose level and strains of mice = 1.92

Level of Significance at 5%
$\alpha = 0.05$, $F_\alpha(V_1,V_2)$ where V_1 is the degree of freedom for A, B, and AB respectively and $V_2$is the degree of freedom for error.
For the main effect A (dose level) and main effect B (strains of mice) $F_{0.05}(2,9)= 4.26$
For the interaction effect AB $F_{0.05}(4,9)= 3.63$

Decision Rule: Reject H_O if F-ratio $\geq F_\alpha(V_1,V_2)$, otherwise we fail to reject H_O.

Decision:
For the main effect A (dose level): Since 23.89 > 4.26, therefore H_O is rejected
For main effect B (strains of mice): Since 3.63 < 4.26, fail to reject H_O
For interaction effect AB (dose level × strains of mice): Since 1.92 < 3.63, therefore H_O is not rejected.

CONCLUSION

The analysis on the Red Blood Cell (RBC) response of the three strains of mice when they are dosed with control, chloramphenicol at low level (500mg/kg) and high level (2500mg/kg) shows that there is no interaction effect between the strains of mice and the three dose level. Also, the three strains of mice and dose level are not significantly different from each other. For the White Blood Cell (WBC) response, the result shows that there is a significant difference among the three dose level and there is no interaction effect between the strains of mice and the dose level and the three strains of mice are not significantly different from each other. The analysis on weight gain of three strains of mice dosed with control, low level (450mg/kg) and high level (2400mg/kg) of chloramphenicol shows that the main effect (A), dose level are not different significantly (i.e. the same) whole the main effect (B), strains of mice are different significantly (they are not the same). There is no interaction effect between the three dose level and strains of mice at 5% level of significance.

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