Polish Journal of Occupational Medicine Vol. 3, No. 2, 201—207, 1990

STATISTICAL ANALYSIS OF THE GENOTOXICITY STUDY RESULTS

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Key words: Micronucleus test, Genotoxicity, Polychromatic erythrocytes, Normochromatic erythrocytes, Approximate normal test, Kastenbaum & Bowman test

Abstract. One of the methods used in the genotoxicity study of chemical compounds is micronucleus test *in vivo*. There is no clear consensus as to which statistical method is most suitable to study the frequency of occurrence of micronucleated polychromatic erythrocytes. This paper proposes two statistical tests: approximate normal test and conditional binomial test (Kastenbaum & Bowman).

INTRODUCTION

It has long been known that some chemicals can cause alternations in genetic material leading to malignant diseases (11). Numerous short-term tests have been developed to detect the ability of chemicals to cause such changes. They are successfully used on a large scale. One of these methods is a micronucleus test *in vivo*. Micronucleus assay is used to identify clastogenic compounds, i.e. those capable of inducing structural changes in chromosomes. The method also detects damages of the mitotic apparatus induced by chemicals (6). The test has specificity (ability to correct classification of non-carcinogenic compounds as negative) of about 80% and sensitivity (ability to correct classification of carcinogenic chemicals as positive) of 54% (3).

A detailed description of the technique has been presented previously (16). In general the method involves the following procedures: animals are exposed to the substance by an appropriate route. They are sacrificed, the bone marrow extracted and smear preparation made and stained. At least 1000 polychromatic erythrocytes per animal are scored for the incidence of micronuclei (genotoxicity assessment) and also the ratio of polychromatic to normochromatic erythrocytes is deter-

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mined (cytotoxicity assay) for each animal by counting a total of 200 erythrocytes.

Several criteria are used for determining positive results. One such result is a statistically significant ($p < 0.05$) dose-related increase in the number of micronucleated polychromatic erythrocytes. Another criterion may be based upon detection of a reproducible and statistically significant positive response to, a least, one of the test points (30, 48, 72 h) after the first of two (0 and 24 h) injections of studied compounds. A test substance producing neither statistically significant dose-related increase in the number of micronucleated polychromatic erythrocytes nor statistically significant and reproducible positive response at any one of the test points was considered non-mutagenic in this assay.

Although a number of statistical procedures have been proposed for the analysis of results obtained in the micronucleus assay, there is as yet no general consensus as to which is the most appropriate. In this paper, some of these methods are discussed and a proposition concerned with statistical analysis of results in the micronucleus test is presented.

I. Comparison of frequency of occurrence of micronucleated polychromatic erythrocytes in the groups under treatment and in the negative controls.

In the literature there are two general approaches toward micronucleated polychromatic erythrocytes. Mitchell & Brice (13), Mitchell et al. (14) prefer nonparametric methods, while other authors are for parametric methods. Frei & Wurgler (4), MacGregor et al. (7), Mackey & MacGregor (9), Margolin, Collins and Mason (10) assume that the probability distribution of the variable under study is known.

Nonparametric methods are slightly weaker to parametric but they do not require the knowledge of probability distributions. The Kolmogorov-Smirnov test was proposed by Mitchell & Brice (13). Those authors applied the nonparametric Kolmogorov-Smirnov two-sample test to 10—12 animal samples achieving correct results. However, the Kolmogorov-Smirnov test may produce incorrect results for four animal samples in our micronucleus assay. The same limitation refers to the Wilcoxon test. Furthermore, there exists another limitation of the Wilcoxon test, namely there are many tied ranks within small samples. Therefore, in our opinion parametric tests are more suitable than nonparamtetric ones.

MacGregor et al. (6) ascertain the estimation that the retaining spontaneous results (negative control) is conclusive in the discussion

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about statistical methods. Hart and Engberg-Pedersen (5) show that the ratio of normochromatic to polichromatic erythrocytes followed a normal distribution, while the incidence of micronucleated polychromatic and normochromatic erythrocytes followed a binomial distribution. Amphlett & Delow (1) assume that the number of micronucleated polychromatic erythrocytes in 1000 polychromatic erythrocytes from each treated group and the negative controls come from Poisson distribution. However, at very low incidence (about 2/1000) the binomial and Poisson distributions are fairly similar (5), and such frequencies (2/1000—5/1000) occur in practice.

In this paper two statistical tests are proposed. The first test is based on a standard normal approximation to the distribution of the difference between two sample proportions (10) and the other — on the conditional binomial test according to Kastenbaum and Bowman (4).

Approximate normal test

This test requires following assumptions:

1° Group sample sizes greater than 500.

2° Probability less than 0.05 for a binary observation from any experimental unit being "positive".

3° Historical control data will not be included in the statistical analysis.

Let N_c and N_t be the sample size in the control and the treated groups respectively and n_c and n_t represent, respectively, the observed numbers of genotoxic events in the control and the treated groups, and n_c/N_c, n_t/N_t represent the associatied observed genotoxic change frequencies (Table 1).

TABLE 1. Binomial results from a mutagenicity experiment

Genotoxic events	Control group	Treated group	Totals
+	n_c	N_t-n_t	n
−	N_c-n_c	N_t-n_t	N−n
Totals	N_c	n_t	N

Experimental units in the control and treated groups may be thought of as having unobservable probabilities P_c and P_t, respectively. We have two alternative hypotheses: H_0: $P_t < P_c$ and H_1: $P_t > P_c$. Statistics for testing H_0 against H_1 is following:

$$Zn = \Delta/SE$$

where,

$$\Delta = (n_t/N_t) - (n_c/N_c)$$

$$SE = [\hat{p}(1-\hat{p})\{1/N_c + 1/N_t\}]^{1/2}$$

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$$\hat{p} = (n_t+n_c)/(N_t+N_c) = n/N.$$

Statistic Zn has the approximately standard normal distribution and one can find probability

$$P_i = \Phi(Z_n)$$

where Φ is the probility distribution function of normal distribution. If $P < \alpha$, H_0 is rejected, where α is the level of significance, if $P > \alpha$, H_0 is accepted.

Conditional binomial test

In this test the alternative hypothesis postulates *a priori* that the treatment results in m time increased genotoxic events frequency comparing to the level of spontaneous frequency.

Testing against the null hypothesis (H_0) at the level α and against the alternative hypothesis (H_1) at the level β leads to the error probabilities shown in Table 2.

TABLE 2. Possible diagnoses as a result of the testing problem (H_0, H_1) solution

Hypotheses	H_1 accepted $1-\beta$	H_1 rejected β
H_0 accepted $1-\alpha$	Inconclusive $P=(1-\alpha)(1-\beta)$	Negative $P=(1-\alpha)\beta$
H_0 rejected α	Positive $P=\alpha(1-\beta)$	Weak positive $P=\alpha\beta$

Let:

$p_0 = N_c/(N_c+N_t)$ $\quad$ $q_0 = N_t/(N_c+N_t)$

$p_1 = N_c/(N_c+mN_t)$ $\quad$ $q_1 = mN_t/(N_c+mN_t)$.

We reject H_0 in the binomal test if

$$P_0 = \sum_{i=0}^{nc} \binom{n}{i} p_0 i\, q_0^{n-i} = \sum_{r=nt}^{n} \binom{n}{r} q_0^r\, p_0^{n-r} < \alpha$$

and, by analogy, we reject H_1 if

$$P_1 = \sum_{i=0}^{nt} \binom{n}{i} i_{q_1}\, p_1^{n-i} = \sum_{r=nc}^{r} \binom{n}{r} p_1^r\, q_1^{n-r} < \beta$$

Example (Table 3).

Both tests, i.e. the approximate normal test and the conditional binomial test, lead to the same conclusion. Under Anionic Brown GM none of the hypotheses H_0 and H_1 can be rejected. Under Direct Yellow 12 both tests reject H_0 and the conditional binomial test accepts H_1,

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TABLE 3. The results of genotoxicity study of Anionic Brown GM (colour index 80, 7—16) and Direct Yellow 12 at dose 40% LD_{50} for male mice. Number of micronucleated polychromatic erythrocytes per 1000 polychromatic erythrocytes

Animal number	Anionic Brown GM		Direct Yellow 12	
	Negative control	Treated group	Negative control	Treated group
1	3	3	6	23
2	2	4	7	24
3	2	4	6	24
4	2	3	7	29
Totals	9	14	26	100
Results of approximate normal test				
$\triangle$ =	(14−9)/4000 = 0.00125		(100−26)/4000 =	0.0185
p =	23/8000 = 0.002875		126/8000 =	0.01575
SE =	0.0012			0.000815
Zn =	0.0125/0.0012 = 1.04		0.0185/0.000815 =	22.7
P =	0.2323			0.0000
Results of conditional binomial test				
(m = 2)				
P_0 = 0.202				0.0000
P_1 = 0.348				0.99941
(m = 1)				
P_0 = 0.202				
P_1 = 0.895				

i.e. Direct Yellow 12 is genotoxic. The binomial test leads to stronger conclusion: frequency of micronucleated erythrocytes is 2 times higher than spontaneous one.

II. Analysis of more than one treated group

In the case of a few treated groups many researchers use analysis of variance with multiple comparisons test as a method of statistical analysis of outcomes (e.g. The Collaborative Study Group (2), McGregor et al. (7). Mitchell (12), Whorton (17). They use regression methods for fitting dose-response relationships (e.g. Amphlett & Delow (1).

Because we do not fit dose-response relationships, we use one-way analysis of variance with multiple comparison tests (Dunnett or Scheffe or Tukey test) to study a few groups of outcomes.

Whorton (17) suggests to use transformation 1/2 ($\sqrt{x}+\sqrt{x+1}$) to stabilize the variance. x denotes number of mutated cells among study group of cells. This transformation really diminishes the variance heterogeneity (Table 4), but sometimes group variances are heterogeneous after transformation and we are not able to use F statistics in analysis of variance (Table 4).

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TABLE 4. The resutls of genotoxicity study of Anionic Brown GM at doses 40% LD_{50} and 80% LD_{50} for male mice

Group of animals	Number of micronucleated/1000 polychromatic erythrocytes	
	before transformation	after transformation
Negative control	3	1.87
	2	1.57
	2	1.57
	2	1.57
Mean	2.25	1.65
SD	0.50	0.15
40% LD_{50}	3	1.87
	4	2.12
	4	2.12
	3	1.87
Mean	3.50	2.00
SD	0.58	0.14
80% LD_{50}	2	1.57
	4	2.12
	4.5	2.23
	3	1.87
Mean	3.38	1.95
SD	1.11	0.29
Homogeneity variance Bertlett's test	2.004	1.796
Probability for Bertlett's test	0.367	0.407

And so the following sugestion appears: after transformation 1/2 ($\sqrt{x}+\sqrt{x+1}$) one ought to use Bartlett test for studying hemogeneity of variance and then the significance of group difference test by parametric (F-Snedocor statistics) or nonparametric (Kruskal-Wallis) analysis of variance with multiple comparisons.

CONCLUSIONS

Approximate normal test and conditional binomial test are applicable to comparison of occurrence of micronucleated polychromatic erythrocytes between the treated group and the negative control. In the case of more than one treated group, we propose to use complete (i.e. with heterogeneity of variance and multiple comparison tests) one-way analysis of variance after Whorton's transformation.

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Received for publication: April 17, 1990
Accepted for publication: May 28, 1990

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