遗传毒性研究

小鼠骨髓微核实验

环境健康学系

微核试验（简称：MNT)发展历史

1959年	Evans等	蚕豆根端细胞电脑辐射暴露
1970年	Boller和Schmid	抗肿瘤药三亚胺醌给药中国黄金地鼠，正式命名MNT
1976年	Countryman，Heddle	人外周血培养淋巴细胞进行MNT
1979年	Cole，King和Wild等	妊娠雌鼠给药，胎仔小鼠的肝细胞和外周血出现微核
1980年	MacGregor等	小鼠外周血检测MN方法
1981年和1983年	Lahdetie,Tates等	精母细胞MNT，预测生殖毒性
1983年	Hayashi, MacGregor等	荧光染料AO特异性染色引入MNT

指导原则

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

MAMMALIAN ERYTHROCYTE MICRONUCLEUS TEST

474

Adopted:
29 July 2016

INTRODUCTION

1. The OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress, changing regulatory needs and animal welfare considerations. The original Test Guideline 474 was adopted in 1983. In 1997, a revised version was adopted, based on scientific progress made to that date. This modified version of the Test Guideline reflects scientific knowledge from more than thirty years of experience with this assay and the interpretation of the data, and in particular the advances in automated scoring technologies and the potential for integrating or combining this test with other general toxicity or genotoxicity studies. This Test Guideline is part of a series of Test Guidelines on genetic toxicology. A document presented as an Introduction to the Test Guidelines on genetic toxicology (1) can also be referred to and provides succinct and useful guidance to users of these Test Guidelines.

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Mammalian Erythrocyte Micronucleus Test

INTRODUCTION

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Office of Food Additive Safety

Redbook 2000

Toxicological Principles for the Safety Assessment of Food Ingredients

July 2000; Updated October 2001, November 2003, & April 2004*

Intro

This guidance represents the Food
thinking on this topic. It does not c
and does not operate to bind FDA
approach if such an approach satisf
and regulations. If you want to disc
staff responsible for implementing
301-436-1200). Contact informatio
For questions regarding the use or
printed copies of individual chapte
Additive Safety (OFAS).

IV.C.1.d. Mammalian Erythrocyte Micronucleus Test

I. Introduction

Micronuclei are cytoplasmic chromatin-containing bodies formed when acentric chromosome fragments or chromosomes lag during anaphase and fail to become incorporated into daughter cell nuclei during cell division. Because genetic damage that results in chromosome breaks, structurally abnormal chromosomes, or spindle abnormalities leads to micronucleus formation, the incidence of micronuclei serves as an index of these types of damage. It has been established that essentially all agents that cause double strand chromosome breaks (clastogens) induce micronuclei. Because enumeration of micronuclei is much faster and less technically demanding than is scoring of chromosomal aberrations, and because micronuclei arise from two important types of genetic damage (clastogenesis and spindle disruption), the micronucleus assay has been widely used to screen for chemicals that cause these types of damage.

4. RECOMMENDATIONS FOR *IN VIVO* TESTS

4.1 Tests for the Detection of Chromosome Damage *In Vivo*

Either the analysis of chromosomal aberrations or the measurement of micronucleated polychromatic erythrocytes in bone marrow cells *in vivo* is considered appropriate for the detection of clastogens. Both rats and mice are considered appropriate for use in the bone marrow micronucleus test. Micronuclei can also be measured in immature (e.g., polychromatic) erythrocytes in peripheral blood in the mouse, or in the newly formed reticulocytes in rat blood (Note 3). Likewise, immature erythrocytes can be used from any other species which has shown an adequate sensitivity to detect clastogens/aneuploidy inducers in bone marrow or peripheral blood (Note 3). Systems for automated analysis (image analysis and flow cytometry) can be used if appropriately validated (OECD, 1997; Hayashi et al., 2000; 2007). Chromosomal aberrations can also be analyzed in peripheral lymphocytes cultured from treated rodents (Note 11).

4.2 Other *In Vivo* Genotoxicity Tests

The same *in vivo* tests described as the second
can be used as follow-up tests to develop weigh
vitro or *in vivo* assays (Notes 11 and 12). Whil
knowledge of the mechanism can help guide the
chromosomal aberrations or of gene mutations i
standard methods in most tissues. Although m
in rodents, this entails prolonged treatment
expression, fixation and accumulation, especially
12). Thus the second *in vivo* assay will often
surrogate. Assays with the most published exp

ICH HARMONISED TRIPARTITE GUIDELINE

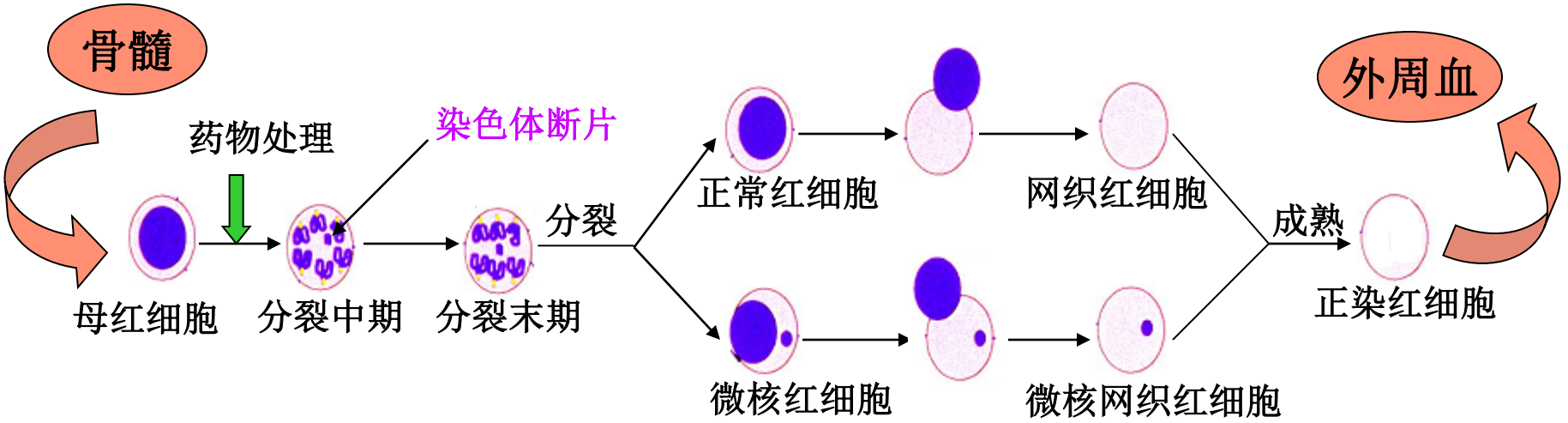
GUIDANCE ON GENOTOXICITY TESTING AND DATA INTERPRETATION FOR PHARMACEUTICALS INTENDED FOR HUMAN USE

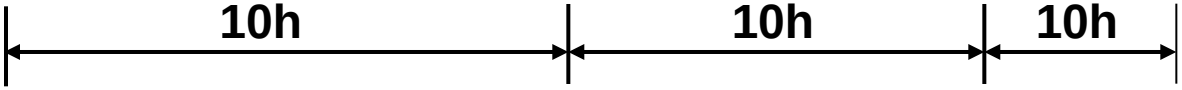
S2(R1)

实验原理

- 是染色体或染色单体的无着丝点断片或纺锤丝受损伤而丢失的整个染色体。
- 可检出DNA断裂剂和非整倍体诱变剂。

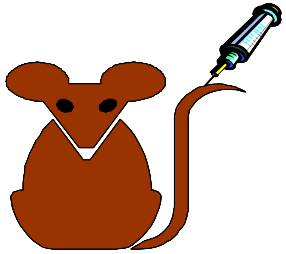
微核细胞形成过程



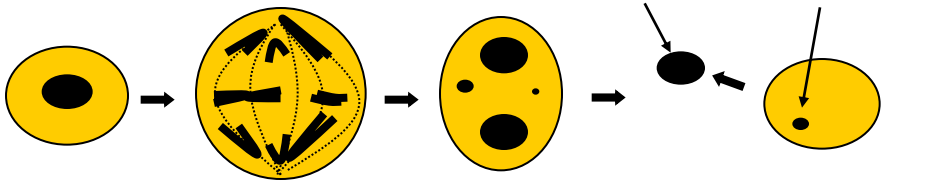


骨髓中检测到微核在18-30h（给药2次，通常在18-24h采样）

外周血液中检测到微核要>30h

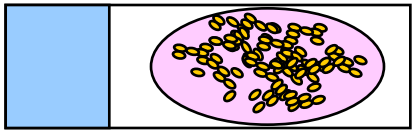


Rats/mice dosed with compound, 3 doses, 6 animals / group. Animals sacrificed 24 or 48 hours later

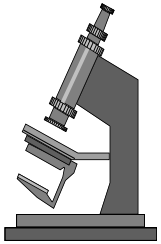


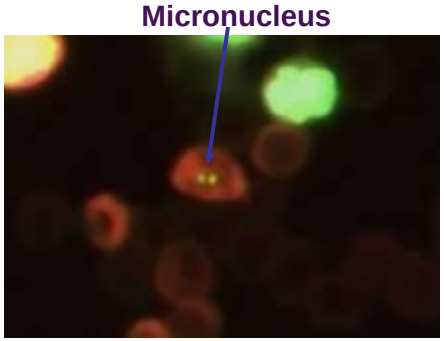
Micronuclei may be formed by loss of whole chromosome during division or by chromosome breakage. The erythrocyte's nucleus is extruded leaving any micronuclei behind

Femurs removed and bone marrow aspirated



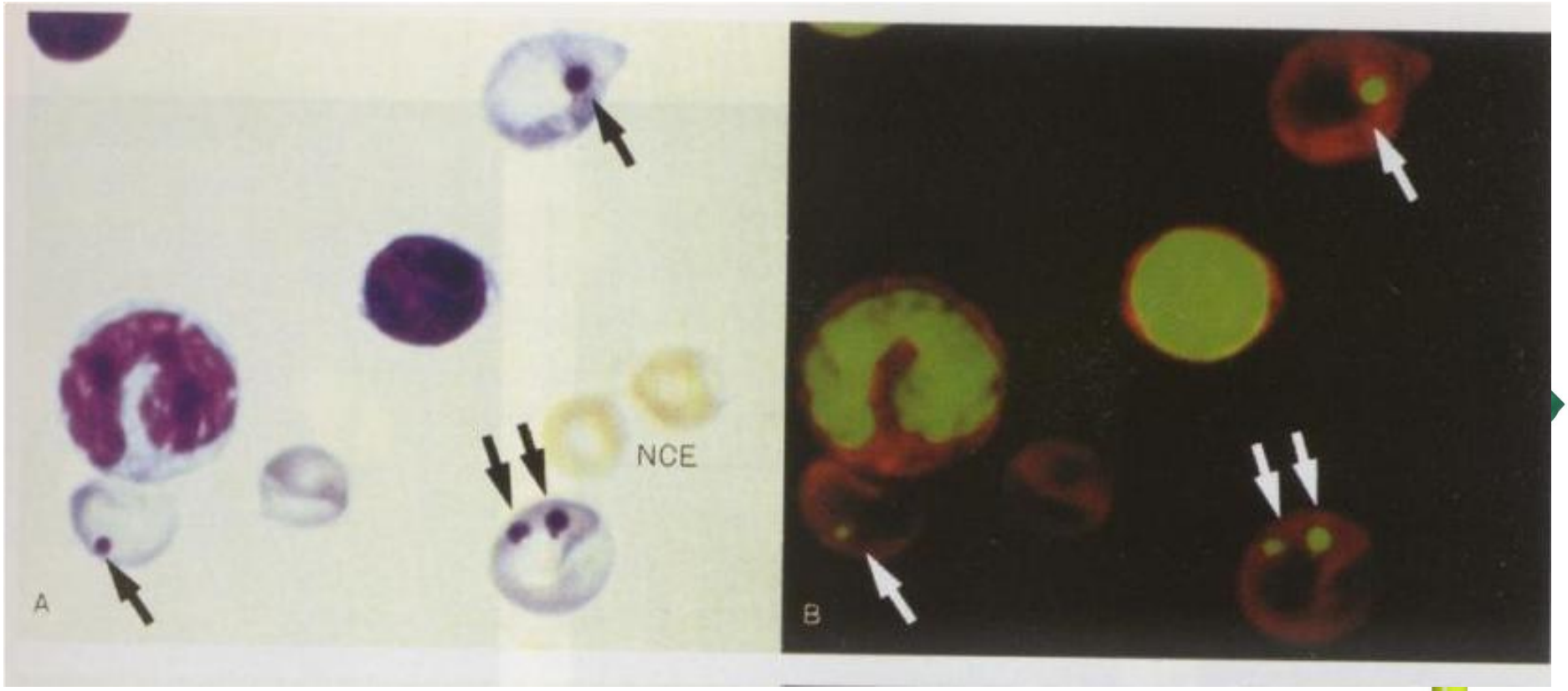
Bone marrow cells spread onto slides. Slides fixed and stained (acridine orange)





2000 cells analysed per animal, number of micronucleated immature erythrocytes scored

Rodent bone-marrow micronucleus test



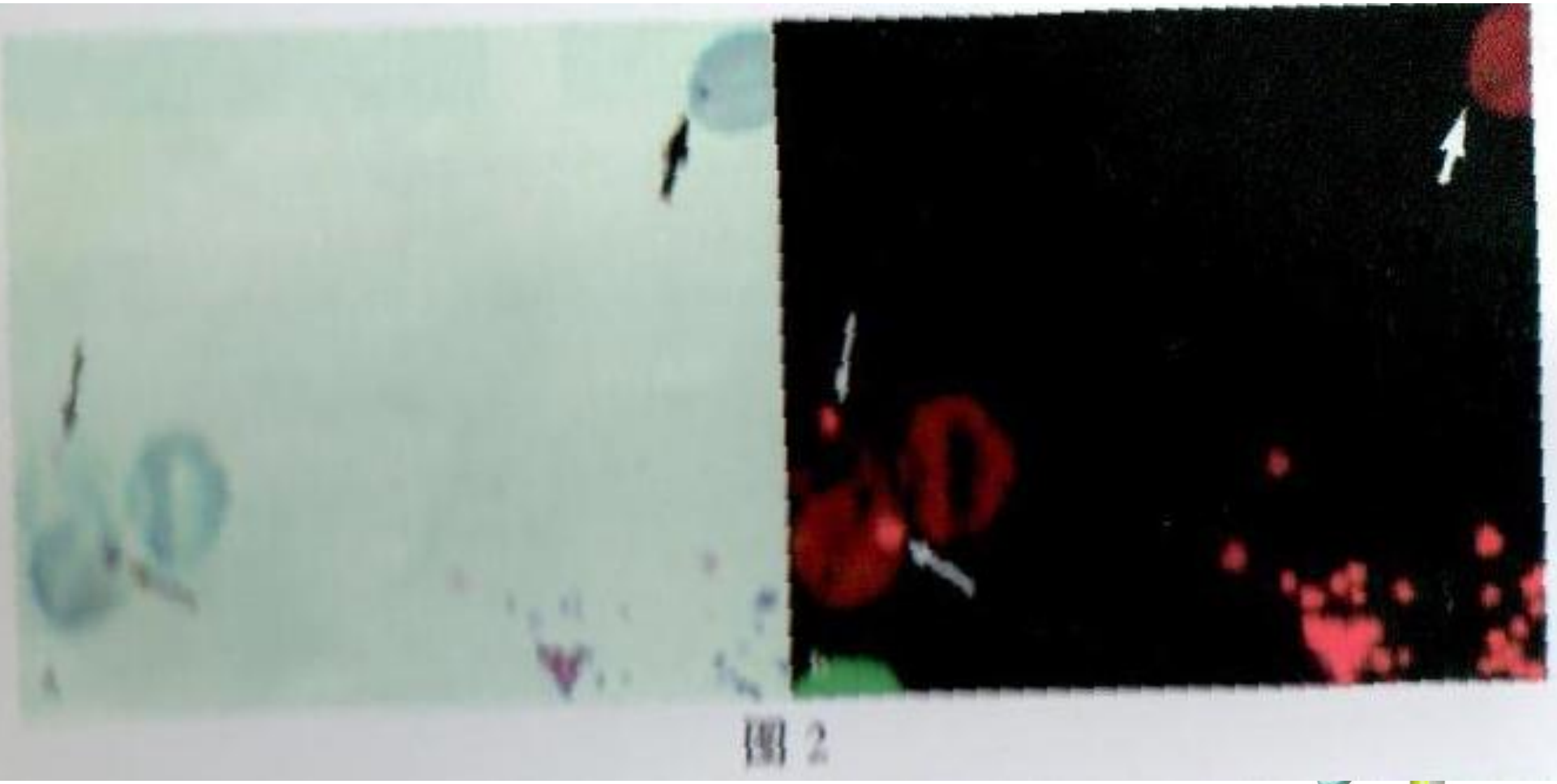


图 2

吖啶橙染色后显示为肥大颗粒

常规微核实验的优缺点

优点	缺点
任何核型的生物材料均可使用	不能判定染色体异常的种类
试验结果可信度高	可能出现伪微核及假阳性结果
可长时间检出染色体异常效应	
可以检出纺锤体毒剂	
易于观察，标本背景清楚	
不需要被检化合物以外的化学物质	
经济、简便、快速	

实验设计

- 剂量选择

- 至少应设置3个剂量组，根据相关毒性试验或预试验的结果确定高剂量，高剂量应产生一定的毒性症状或骨髓毒性（如嗜多染红细胞在红细胞总数中的比例降低）。
- 对于低毒性化合物，给药时间≤14天的推荐最高剂量为2000mg/kg/d，给药时间>14天的推荐最高剂量为1000mg/kg/d；对于单次给药或一天内多次给药达2000mg/kg/d仍无毒性的化合物，设置3个剂量组的意义不大。

实验设计

➢ 动物选择

- 品系：合适品系的小鼠均可使用，本实验室常采用ICR小鼠。
- 周龄：给药时7－8周龄。
- 使用动物数与性别：一般使用雄性小鼠，每组至少6只。

若性别间存在明显的毒性或代谢方面的差异，则采用两种性别的动物，每组雌雄至少各5只。

如果受试物专用于一种性别，则选用相应性别的动物进行试验。

实验设计

- 对照品

- 空白对照品，一般为水或生理盐水。
- 溶媒对照品，采用供试品配制使用的溶媒，若溶媒对照品为常用无毒性的对照品，如**HPMC、CMC**等，则无需空白对照品。
- 阳性对照品，本实验一般使用环磷酰胺，剂量在**50~100 mg/kg**。

实验设计

- 给药

- 给药频率

短期实验通常采用给药1~3次；或者整合在重复给药实验中。给药间隔通常约24小时。

- 给药途径

供试品的给药途径尽可能与临床拟用途径相同，一般采用灌胃和腹腔注射。

阴性对照物与供试品给药途径一致。

阳性对照物的给药途径可以不同于供试品。实验室一般阳性对照物以灌胃给药方式给予环磷酰胺。

实验设计

- 临床观察

  给药期需观察小鼠临床症状。

- 骨髓标本采样

  采用单次给药，一般在给药后24~48小时至少采样2次。

  采用短期实验进行给药（≥2次给药）和重复给药实验，一般在末次给药后18~24小时采样1次。

- 阅片

  每只动物至少计数200个红细胞以确定PCE和总红细胞的比例（PCE/（PCE+NCE））。

  至少计数2000个嗜多染红细胞以判断嗜多染红细胞的微核率。

本次教学实验目的

- 了解遗传毒理学研究动物体内实验设计基础知识。
- 熟悉小鼠骨髓微核实验流程。
- 掌握小鼠骨髓标本片制作及阅片方法。
- 掌握小鼠骨髓微核实验的数据评价方法。

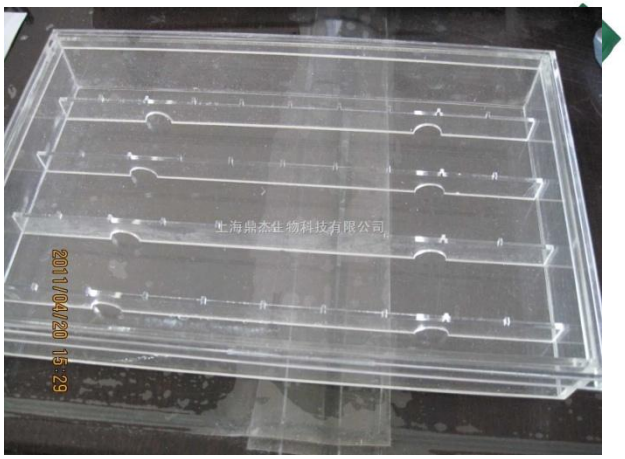
材料和试剂

实验动物的选择

- 健康ICR或者昆明小鼠
- 雄性
- 8周龄（一般订购6-7周龄，适应性饲养后为8周龄。）
- 体重（30±5g）
- 适应性喂养
- 禁食，不禁水（不超过18 h）

实验器材

- 称鼠天平
- 制标本片用—注射器（1mL或2mL）、手术剪、止血钳、小镊子、滤纸、铅笔、纱布（小块）和玻片（带磨砂区域，可标记）。
- 标本片染色+阅片用—离心管、烧杯、染色缸、湿盒、盖玻片、擦镜纸等。



实验试剂

- 制作标本片用—牛血清
- 标本片染色用—吉姆萨染液，磷酸缓冲液，柠檬酸溶液
- 封片用—二甲苯、中性树胶
- 阅片用—香柏油、无水乙醇

本次教学实验剂量设计（四个化合物）

举例

受试物1：环磷酰胺，**Cyclophosphamide ， CP**

CAS no. 50-18-0 (CAS no. 6055-19-2)]

理化性质：白色晶体状颗粒，易溶于水。

- 空白对照：蒸馏水
- **Dose 1= 25 mg/kg**
- **Dose 2= 50 mg/kg**
- **Dose 3 = 100 mg/kg**
- ……选择2~4个剂量组进行试验。

给药:按0.2ml/10g体重进行给药

给药剂量 (mg/kg)	供试品溶液的浓度 (mg/mL)
?	?
?	?
?	?

实验流程

小鼠称体重

给药体积计算

灌胃操作

给药当天临床症状观察

12月26日，Day1

解剖前中毒症状观察、处死小鼠制作骨髓涂片

试剂配制，标本片染色，封片

阅片，计算微核率

12月27日，Day2

编号标记

有哪些标记方法？

- 记号笔尾部标记。

  适用于短期标记1~2天，长期实验则需要用记号笔继续加深。

- 苦味酸酒精饱和溶液

  黄色（标记，1–9）

- 0.5%品红溶液

  红色（标记，10，20，30…）

动物给药

- 根据选择的给药体积和每只小鼠的体重计算给药量。
- 根据给药剂量取相应的受试物溶液进行给药。

注意事项

- 注射器吸取液体时，不能有气泡，否则影响剂量的准确性。
- 每个不同剂量的溶液吸完以后，用下一个剂量润洗。
- 注射器的最小刻度，四舍五入后，按实际给药量记录。

中毒症状的观察

- 动物给药时间，死亡时间
- 仔细观察中毒症状，持续和/或恢复时间。
- 各剂量组动物的死亡数。

标本制作与染色

➢ 骨髓细胞制备和涂片

- 剪取股骨，用干净纱布擦拭，剔去肌肉，剪去每节骨骺端，用止血钳挤出骨髓液置于载玻片一端。
- 滴加牛血清，取另一张载玻片以打圈方式将骨髓液与血清混匀，以约45°角度将骨髓悬浊液平稳均匀地向载玻片的另一端推动，一般3~5cm长度为宜。尽量使骨髓涂片的涂膜薄且均匀，细胞分布均匀。

➢ 固定

骨髓涂片干透，滴加甲醇溶液覆盖整个涂膜，自然风干。

标本制作与染色

- 染色

Giemsa染色液用磷酸缓冲液配制成稀释液，一般浓度为20-30％ (v/v)。

若染色效果不够明显区分正染红细胞(NCE)和嗜多染红细胞(PCE)，可用0.004%的柠檬酸液处理数秒钟。

- 封片

二甲苯溶液中浸泡2~5分钟后取出，在二甲苯还未干透时滴少量中性树胶，用小镊子夹取盖玻片，盖上盖玻片，避免产生气泡，使中性树胶分布均匀。

阅片

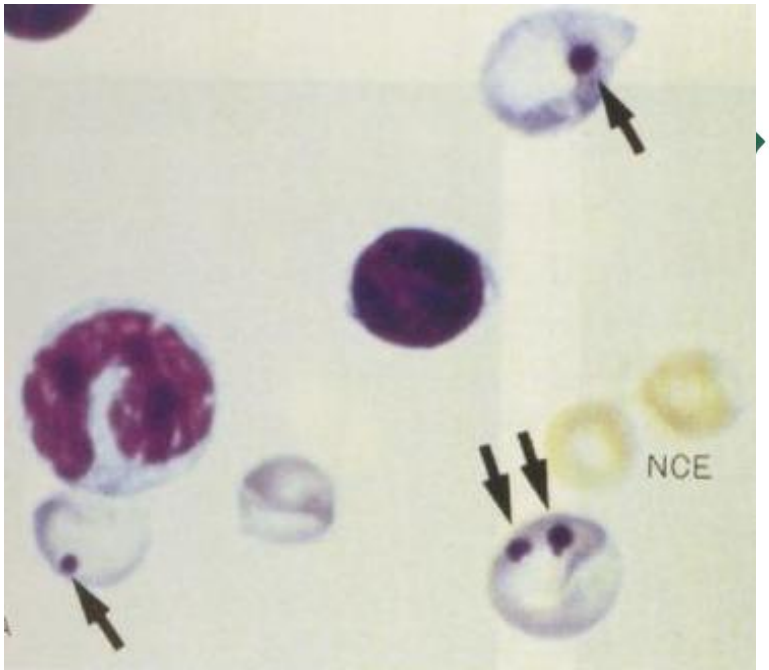
➢ 视野选择及计数

- 先以低倍镜粗检，选择细胞分布均匀、疏密适度、形态完整、染色良好的区域，共挑选4处这样的区域；
- 高倍镜（100x 油镜)，在标本片上滴上香柏油观察。按一定顺序进行PCE和微核计数。每个区域计数约500个PCE。
- 每只动物至少计数200个红细胞以确定PCE和总红细胞的比例（PCE/（PCE+NCE））。
- 至少计数2000个嗜多染红细胞以判断嗜多染红细胞的微核率。

阅片

- **PCE与NCE的鉴别**

PCE是刚排核的红细胞。由于PCE中含有核糖体，Giemsa染色呈灰蓝色。成熟的红细胞（即NCE）的核糖体已溶解，染色成橘红色。但每一批样本，由于各种因素的影响，染色深浅不完全一致。因此在计数时必须区分PCE与NCE的差别。



阅片

➢ 微核的判别标准

- 色彩：PCE中微核的嗜色性与核质一致，呈紫红色或蓝紫色。
- 形态：典型的微核呈圆形，边缘光滑整齐，偶尔也可呈椭圆形、肾形、马蹄形及环形。
- 大小：上限为NCE直径的一半，下限则在能观察到的范围内。通常为红细胞的1/20-1/5。
- 轮廓：微核有清楚的核膜包饶，边缘光滑周围是白色时，不是微核的可能性很大。
- 聚焦：微核是位于细胞内的颗粒，将显微镜的焦距上下移动以确认。如果上下调焦距颗粒发白光，则不是微核。

实验报告的填写