M.PHARM (PHARMACOLOGY) 2nd SEMESTER

ACUTE EYE IRRITATION

INTRODUCTION

- **Eye irritation** refers to the production of changes in the eye, which are fully reversible, occurring after the exposure of the eye to a chemical or mixture.
- This Test Guideline 405 was adopted in 1981 and updated in 1987, 2002, 2020, and 2021.
- Testing in animals should only be conducted, if necessary, after considering available alternative methods and using appropriate ones.
- The latest update mainly focused on the use of analgesics and anesthetics without impacting the basic concept and structure of the Test Guideline.
- Topical anesthetics, systemic analgesics, and humane endpoints should be routinely used during acute eye irritation and corrosion in vivo testing.
- Balanced preemptive pain management includes:
  - routine pretreatment with a topical anesthetic (e.g., proparacaine or tetracaine) and a systemic analgesic (e.g., buprenorphine),
  - routine post-treatment schedule of systemic analgesia (e.g., buprenorphine and meloxicam),
  - scheduled observation, monitoring, and recording of animals for clinical signs of pain,
  - scheduled observation, monitoring, and recording of all eye injuries' nature, severity, and progression.

PRINCIPLE OF THE IN VIVO TEST

- Pretreatment with a systemic analgesic and induction of appropriate topical anesthesia,
  - The substance to be tested is applied in a single dose to one of the eyes of the experimental animal; the untreated eye serves as the control.

- The degree of eye irritation is evaluated by scoring lesions of the conjunctiva, cornea, and iris.
- The duration of the study should be sufficient to evaluate the reversibility or irreversibility of the effects.
- Animals showing signs of severe pain at any stage of the test consistent with the humane endpoints described in this Test Guideline should be humanely sacrificed, and the substance assessed accordingly.

ELEMENTS FOREYE IRRITATION TEST

- Selection of Species - Preferably young & healthy Albino Rabbit.
- Prep. of animal - Both eyes of each animal should be examined within 24 hours before testing starts. Animals showing eye irritation, ocular defects, or pre-existing corneal injury should not be used.
- Housing and feeding conditions.
  - Temperature = 20 C (± 3^{o} C)
  - Relative humidity= 30-70%
  - Lighting is artificial= 12 hours light, 12 hours dark
  - Feeding= conventional diets with unlimited drinking water.

TEST PROCEDURE

Use of topical anesthetics and systemic analgesics -

- 60 min. prior to test substance application (TSA), buprenorphine 0.01 mg/kg is administered by subcutaneous injection (SC) to provide a therapeutic level of systemic analgesia. They do not alter ocular responses.

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- 5 min. prior to TSA, 1 or 2 drops of a topical ocular anesthetic (e.g., 0.5% proparacaine hydrochloride) are applied to each eye.

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- If the test substance is anticipated to cause significant pain, it should not normally be tested in vivo. However, in case of testing is necessary, consideration should be given to additional applications of the topical anesthetic at 5-minute intervals prior to TSA.

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- Multiple applications of topical anesthetics could potentially cause a slight increase in the severity and time required for chemically induced lesions to clear.

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- 8 hrs. after TSA, buprenorphine 0.01 mg/kg SC and meloxicam 0.5 mg/kg SC are administered to provide a continued therapeutic level of systemic analgesia. Meloxicam should not be administered until at least 8 hrs. after TSA to avoid any possible interference with the study.

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- After the initial 8-hour post-TSA treatment, buprenorphine 0.01 mg/kg SC should be administered every 12 hours, with meloxicam 0.5 mg/kg SC every 24 hours, until the ocular lesions resolve, and no clinical signs of pain and distress are present.

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- If an animal shows signs of pain and distress during the study, a "rescue" dose of buprenorphine 0.03 mg/kg SC would be given immediately and repeated as often as every 8 hours, if necessary, instead of 0.01 mg/kg SC every 12 hours.

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- Meloxicam 0.5 mg/kg SC would be administered every 24 hours in conjunction with the "rescue" dose of buprenorphine, but not until at least 8 hours post-TSA.

APPLICATION OF THE TEST SUBSTANCE

- The test substance should be placed in the conjunctival sac of one eye of each animal after gently pulling the lower lid away from the eyeball. The lids are then gently held together for about one second to prevent the loss of the material. The other eye, which remains untreated, serves as a control.
- Eyes are not washed for 24 hrs. of test animal, except for solids and in case of immediate corrosive or irritating effects. At 24 hours a washout may be used.

DOSE LEVEL

Testing of Liquid	Testing of Solid	Testing of Aerosols
A dose of 0.1mL is used	Dose-volume of 0.1 mL or weight of not more than 100 mg is used.	The substance is sprayed onto weighing paper. The weight increase of the paper is used.
Pump sprays should not be used for instilling the substance directly into the eye.	Test material should be ground to fine dust. If a solid test substance has not been removed from the eye by physiological mechanisms in1 hour after treatment,	The test substance was administered to the eye in a simple burst of 1 sec, from 10 cm directly in front of the eye.
The liquid spray should be expelled and collected in a container prior to instilling 0.1 mL into the eye.	then the eye may be rinsed with saline or distilled water.	Care should be taken not to damage the eye from the pressure of the spray.

INITIAL TEST (IN VIVO EYE IRRITATION/CORROSION TEST USING ONE ANIMAL)

- It is strongly recommended that the in vivo test be performed initially using one animal. Observations allow for the determination of severity and reversibility before proceeding to a confirmatory test in a second animal.
- If the results of this test indicate the substance to be corrosive or a severe irritant to the eye, further testing for ocular irritancy should not be performed.

CONFIRMATORY TEST (IN VIVO EYE IRRITATION TEST WITH ADDITIONAL ANIMALS)

- If a corrosive or severe irritant effect is not observed in the initial test, the irritant or negative response should be confirmed using up to two additional animals.
- If an irritant effect is observed in the initial test, it is recommended that the confirmatory test be conducted in a sequential manner in one animal at a time, rather than exposing the two additional animals simultaneously.
- If the second animal reveals corrosive or severe irritant effects, the test is not continued.
- If results from the second animal are sufficient to allow for a hazard classification determination, then no further testing should be conducted.

OBSERVATION PERIOD

- The duration of the observation period should be sufficient to evaluate fully the magnitude and reversibility of the effects observed.
- The experiment should be terminated at any time that the animal shows signs of severe pain or distress.
- To determine the reversibility of effects, the animals should be observed normally for 21 days post-administration of the test substance. If reversibility is seen before 21 days, the experiment should be terminated at that time.

GRADING OF OCULAR LESIONS

1. Cornea

No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal luster); details of iris clearly visible	1
Easily discernible translucent area; details of iris slightly obscured	2
Narcous area; no details of iris visible	3
Opaque cornea; iris not discernible through the opacity	4
*The area of corneal opacity should be noted	

2. Iris

Normal	0
Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperemia; or injection; iris reactive to light	1
Hemorrhage, gross destruction, or no reaction to light	2

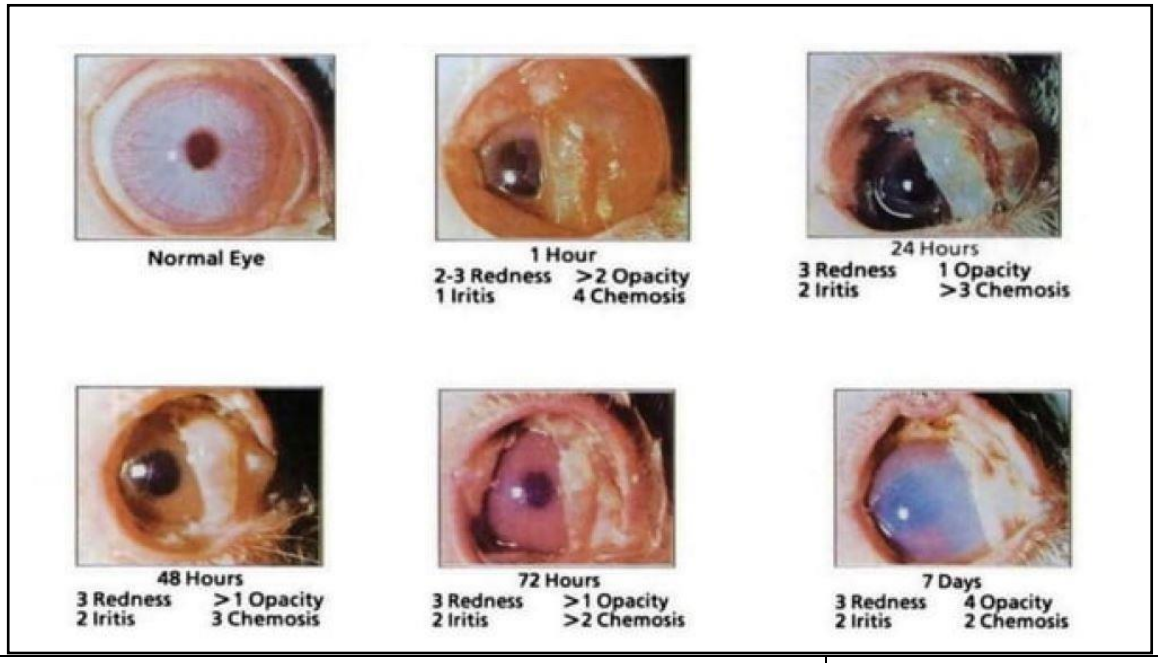
3. Conjunctiva

Normal	0
Some blood vessels hyperemic (injected)	1
Diffuse, crimson color; individual vessels not easily	2

discernible	
Diffuse beefy red	3

4. Chemosis

Normal	0



Some swelling above normal	1
Obvious swelling, with partial eversion of lids	2
Swelling, with lids about half closed	3
Swelling, with lids more than half closed	4

Fig. 1: Ocular Lesions

SKIN SENSITIZATION

INTRODUCTION

- **Skin sensitization** (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance.

- OECD Guidelines for Testing of Chemicals are periodically reviewed considering scientific progress. This updated version of Guideline 406 (originally adopted in 1981, revised in 1991, and in 2021) considers and draws attention to new skin sensitization TestGuidelines.

- In the updated version (17 Jul 1992) preference was given to the guinea pig as an animal of choice for predictive sensitization tests for several decades.

TYPES OF TESTS

The present test guideline describes two types of tests:

- The Guinea pig maximization Test (GPMT) of Magnusson and Kligman which uses adjuvant (Freund's Complete Adjuvant (FCA) to potentiate skin sensitization and,
- The non-adjuvant Buehler Test.

GENERAL PRINCIPLE OF SENSITISATION TESTS IN GUINEA PIGS

- The test animals are initially exposed to the test substance by intradermal injection and/or epidermal application (induction exposure).
- Following a rest period of 10 to 14 days (induction period), during which an immune response may develop, the animals are exposed to a challenge dose.
- The extent and degree of skin reaction to the challenge exposure in the test animals are compared with that demonstrated by control animals that undergo sham treatment during induction and receive the challenge exposure.

ELEMENTS COMMON TO SENSITISATION TESTS IN GUINEA PIGS

- Sex of animals-Healthy Males/females (Females should be non-pregnant).
- Housing and feeding conditions.
    - Temperature = 20 C (± 30º C)
    - Relative humidity= 30-70%
    - Lighting is artificial= 12 hours light, 12 hours dark
    - Feeding conventional diets with unlimited drinking water.
    - It is essential that guinea pigs receive an adequate amount of ascorbic acid.

PREPARATION OF THE ANIMALS

- Animals are acclimatized to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomized and assigned to the treatment groups.
- Removal of hair is by clipping, shaving or possibly by chemical depilation, depending on the test method used.
- Care should be taken to avoid abrading the skin.
- The animals are weighed before the test commences and at the end of the test.

RELIABILITY CHECK

• Sensitivity of the technique used should be assessed every six months by use of substances that are known to have mild-to-moderate skin sensitization properties

- A response of:
    - 30% in an adjuvant test
    - 15% in a non-adjuvant test should be expected for mild/moderate sensitizers.
    - Preferred substances are hexyl cinnamic aldehyde (CAS No. 101-86-0), mercaptobenzothiazole (CAS No. 149-30-4), and benzocaine (CAS No. 94-09-7).

REMOVAL OF THE TEST SUBSTANCE

- This should be achieved using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

1. GUINEA-PIG MAXIMISATION TEST METHOD

Number of animals

- Treatment group = Min. 10 animals
- control group = 5 animals
- When it is not possible to conclude that the test substance is a sensitizer, testing in additional animals to give a total of at least 20 tests and 10 control animals is strongly recommended.

Dose levels

- The conc. of test substance used for each induction exposure should be well-tolerated systemically and should be the highest to cause mild-to-moderate skin irritation.
- The conc. used for the challenge exposure should be the highest non-irritant dose.
- The appropriate conc. can be determined from a pilot study using 2 or 3 animals. Consideration should be given to the use of FCA-treated animals for this purpose.

INDUCTION: INTRADERMAL INJECTIONS

Day 0-treated group

- 3 pairs of intradermal injections of 0.1 ml volume are given in the shoulder region which is cleared of hair so that one of each pair lies on each side of the midline.

Injection 1:	a 1:1 mixture (v/v) FCA/water or physiological saline
Injection 2:	the test substance in an appropriate vehicle at the selected concentration
Injection 3:	the test substance at the selected concentration formulated in a 1:1 mixture (v/v) FCA/water or physiological saline.

- In injection 3, water-soluble substances are dissolved in the aqueous phase prior to mixing with FCA.
- Liposoluble or insoluble substances are suspended in FCA prior to combining with the aqueous phase.
- The concentration of the test substance shall be equal to that used in injection 2.
- Injections 1 and 2 are given close to each other and nearest the head, while 3 is given towards the caudal part of the test area.

Day 0-control group

- 3 pairs of intradermal injections of 0.1 ml volume are given in the same sites as in the treated animals.

Injection 1:	a 1:1 mixture (v/v) FCA/water or physiological saline
Injection 2:	the undiluted vehicle
Injection 3:	a 50% w/v formulation of the vehicle in a 1:1 mixture (v/v) FCA/water or physiological saline

INDUCTION: TOPICAL APPLICATION

Day 5-7-treated and control groups.

- 24 hours before the topical induction application. The test area, after close-clipping or shaving, is painted with 0.5 ml of 10% sodium lauryl sulfate in Vaseline, to create a local irritation (if the substance is not a skin irritant).

Day 6-8-treated group

- The test area is again cleared of hair.
- A filter paper (2 x 4 cm) is fully loaded with a test substance in a suitable vehicle and applied to the test area for 48 hours.
- The choice of vehicle should be justified.
- Solids are finely pulverized and incorporated in a suitable vehicle.
- Liquids can be applied undiluted, if appropriate.

CHALLENGE: TOPICAL APPLICATION

Day 20-22-treated and control groups.

- The flanks of treated and controlled animals are cleared of hair.
- A patch with the test substance is applied to one flank of the animals and, a patch with the vehicle only may also be applied to the other flank.
- The patches are held in contact by an occlusive dressing for 24 hours.

OBSERVATIONS -treated and control groups.

- Remove the patch after 21 hrs., the challenge area is cleaned and closely clipped or shaved.

- 3 hours later (48 hours from the start of the challenge application) the skin reaction is observed.
- 24 hours after this observation a second observation (72 hours) is made and once again recorded.

Blind reading of test and control animals is encouraged.

EVALUATION OF CHALLENGE PATCH TEST REACTIONS

MAGNUSSON AND KLIGMAN GRADING SCALE

0	No visible change
1	Discrete or patchy erythema
2	Moderate and confluent erythema
3	Intense erythema and swelling

RECHALLENGE

- If it is necessary to clarify the results obtained in the first challenge,a second challenge (i.e., a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one.
- A rechallenge may also be performed on the original control group.

2. THE BUEHLER TEST METHOD

Number of animals

- Treatment group- 20 animals
- Control group- 10 animals

Dose levels

- The conc. of the test substance for each induction, exposure should be the highest to cause mild irritation.
- The conc. used for the challenge exposure should be the highest non-irritating dose.
- The appropriate conc. can be determined from a pilot study using two or three animals.
- Water soluble test materials: water or a dilute non- irritating solution of surfactant as the vehicle.

- For other test materials- 80% ethanol/water is preferred for induction and acetone for the challenge

INDUCTION: TOPICAL APPLICATION

Day 0-treated group

- One flank is cleared of hair (closely clipped).
- The test patch system should be fully loaded with the test substance in a suitable vehicle and held in contact with the skin for 6 hours.

Day 0-control group

- One flank is cleared of hair (closely clipped).
- The vehicle only is applied in a similar manner to that used for the treated group.
- The test patch system is held in contact with the skin for 6 hours.

Days 6-8 and 13-15-treated and control groups.

- The same application as on day 0 is carried out on the same test area (cleared of hair if necessary) of the same flank on days 6-8, and again on days 13-15.

CHALLENGE

Day 27-29-treated and control groups.

- The untreated flank of treated and controlled animals is cleared of hair (closely clipped).
- An occlusive patch of the test substance is applied, at the maximum non-irritant concentration, to the posterior untreated flank of treated and control animals.
- When relevant, an occlusive patch with vehicle only is also applied to the anterior untreated flank of both treated and control animals.
- The patches are held in contact by a suitable dressing for 6 hours.

OBSERVATIONS - treated and control groups.

- Approx. **21 hours** after removing the patch the challenge area is cleared of hair.
    I. Approx. 3 hrs. later (i.e., 30 hrs. after the application of the challenge patch) the skin reactions are observed and recorded.
    II. Approx. 24 hours after the 30-hour observation (54 hours after application of the challenge patch) skin reactions are again observed and recorded.
- Blind reading of test and control animals is encouraged.

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