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THE HEN'S EGG TEST - CHORIO-ALLANTOIC-MEMBRANE (HET -CAM) AND HAEMOLYSIS STUDY AS AN ALTERNATIVE TEST FOR OCULAR IRRITANCY ANIMAL EXPERIMENTS (Draize Test)

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ABSTRACT

Human eyes are frequently exposed to chemicals accidentally or on purpose due to their external location. Therefore, chemicals are required to undergo the evaluation of the ocular irritancy for their safe handling and use before release into the market. Draize rabbit eye test developed in 1944, has been a gold standard test which was enlisted as OECD TG 404 and OECD TG 405 but it has been criticized with respect to animal welfare due to invasive and cruel procedure. To replace it, diverse alternatives have been developed: (i) Hen Egg Test-Chorio-Allantoic-Membrane (HET-CAM) test (ii) RBC Haemolysis study.

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INTRODUCTION

Originally the Hen Egg Test-Chorio-Allantoic-Membrane (HET-CAM) test was developed for toxicity and irritation studies as an alternative method to replace the Draize-Test.

Hen Egg Test-Chorio-Allantoic-Membrane (HET-CAM) test

Ocular irritation of the developed formulation was checked by hen's egg chorioallantoic membrane test which is a rapid, sensitive, and inexpensive test. Testing with an incubated egg is a borderline case between in vivo and in vitro systems and does not conflict with the ethical and legal obligations.^[1,2]

The chorioallantoic membrane of the chick embryo is a complete tissue including veins, arteries, and capillaries and is technically very easy to study.

It responds to injury with a complete inflammatory process, a process similar to that induced in the conjunctival tissue of the rabbit eyes.

Developed formulation was tested by this method and the result was compared with those obtained using normal saline, which was used as control that is supposed to be practically non-irritant. A means score of 0 was obtained for normal saline^[3]

The study shows that the formulation is non-irritant to mild irritant and could be regarded as well tolerated. The irritation potential of an ophthalmic medicine can be quantified using this method, by monitoring damage to blood vessels.^[4,5,6]

Haemolysis study

The blood cells swells when placed in hypotonic solution. While in hypertonic solution shrinkage takes place. Their shapes remain normal in isotonic solution. This principle is used to measure Isotonicity of formulation. One drop of human blood was mixed with one drop of formulation and further incubated for 30 min. and further diluted with saline and observe microscope at resolution of 10X. Similarly 1.8 % and 0.45% and 0.9% Sodium chloride solution were used as hypertonic and hypotonic solution and isotonic solution respectively. The above procedure was carried out using this solution instead of formulation and the appearance of cells was compare with appearance of cell in formulation.

According to Hemolysis study we found that no marked changes in RBC's with our formulation, which is similar to innovator hence we concluded that our formulation is isotonic. Hence in vitro studies with RBC's conclude that our formulation is same as that of innovator. And it is the safe for ophthalmic use without causing any irritation of discomfort hence no need of Bioequivalence

MATERIALS AND METHOD:

RBC received from local pathology lab and Hen eggs (9 days fertilized) were procured from Khadkeshwar Hatcheries Ltd, Aurangabad.

EXPERIMENTAL

Procedure for Hen Egg Test-Chorio-Allantoic-Membrane (HET-CAM) test:

Test system:

In this test fertilized 4 Hens's eggs weighing between 10-60 gms were selected.

Purchased the 4 hen eggs (i.e 9 days fertilized egg) from Khadkeshwar Hatcheries Ltd, Aurangabad.

The eggs are stored in egg boxes (blunt ends upwards).

Selecting the Eggs:

Eggs blunt ends are then (on day 10) illuminated with a Candling lamp.

(Note: Only eggs with an emergent embryonic vascular system are used for further testing. Eggs that have not been fertilized or have not undergone embryo genesis are rejected)

Preparation Of The Eggs

The egg shell is opened along the marked line with the help of spoon and then visible white Membrane is moistened with a few mL of physiological saline.

Test Substance Testing

Test substance (F5 formulation eye drop solution) instilled into egg membrane and same time innovator also instilled into the another egg membrane, simultaneously 0.9% sodium chloride considered as negative control sample and 1.8% Sodium chloride solution considered as Positive control

End Points

End points are Haemorrhage, Vasoconstriction, Coagulation and Lysis and checked after 10 min of solution instillation^[6]

Procedure for The Haemolysis Study**RBC's washing and separation:**

1. Collected the blood sample (1mL) from healthy human and kept in the centrifuge tube.
2. Immediately added the 20 μ L EDTA solution (as an anticoagulants) into above mentioned centrifuge tube and mixed properly.
3. With the help on centrifuge apparatus, centrifuged the step 2 sample for 5 min at 3500 rpm.
4. After completion of centrifugation, blood components were settling down into the bottom and removed the supernant (serum) solution.
5. Added the normal saline solution into blood containing centrifuge tube and centrifuged the samples for 5 min at 3500 rpm. and completed of centrifugation, blood components
6. are settling down into the bottom and supernant (serum) solution discarded and repeated for 3 times
7. RBC's separated and used for further study.

B) Different solution preparation:

1. During this study prepared the hypertonic solution (1.8% NaCl) and Hypotonic solution (0.45% NaCl).
2. Collected the normal saline, RLD or Innovator batch sample and In house ophthalmic solution batches (F5 and F5) sample for this study.
3. From above samples pipetted the 0.5 mL (500 μ L) into test tube and add 10 μ L of RBC's sample, Mixed properly.

Magnification of slide:

1. One drop from each test tube containing solution placed into the glass slide and covered with cover slip
2. Placed a small drop of immersion oil directly into slide over a coverslip.
3. Rotated the 100x objectives into the immersion oil and focus only with fine focus.
4. Similarly one by one each slide magnified in the 100x objectives^[8]

OBSERVATION**Hen Egg Test-Chorio-Allantoic-Membrane (HET-CAM) test**

Candling lamp observation for confirmation of emergent embryonic vascular system.

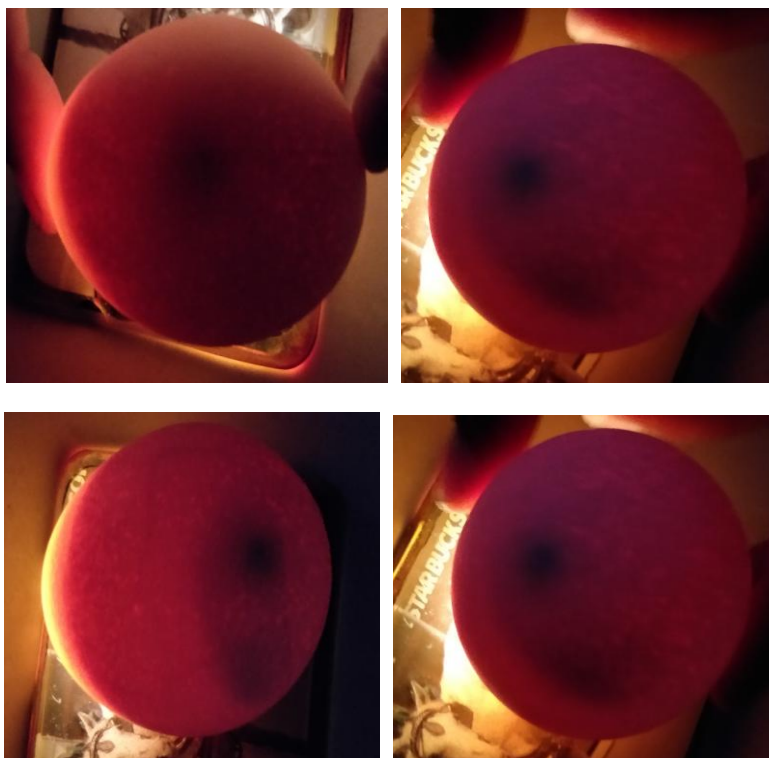


Figure 1 Candling lamp observation for confirmation of emergent embryonic vascular system.

Observation

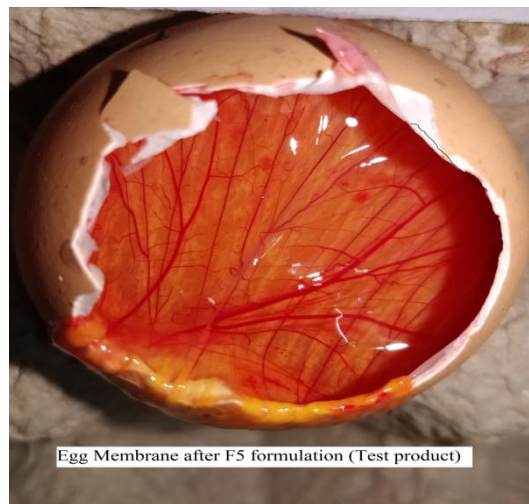


Figure 2 Hen Egg Test-Chorio-Allantoic-Membrane (HET-CAM) test.

OBSERVATION
Haemolysis Study

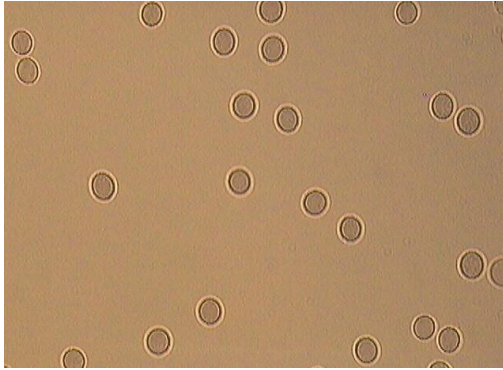


Figure 3 Normal RBC.

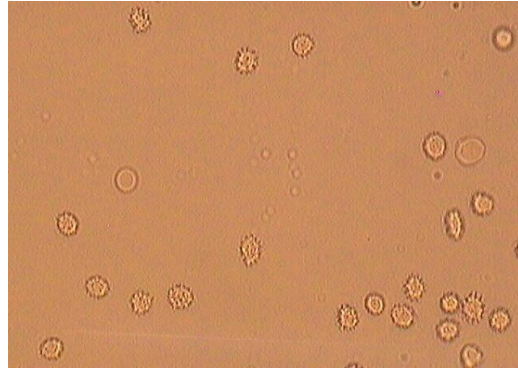


Figure 4 Hypertonic Solution RBC.



Figure 5 Hypotonic solutions RBC.

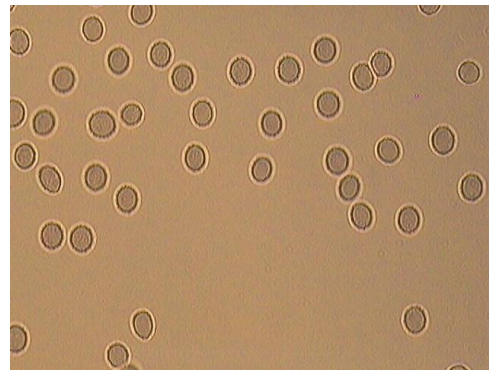
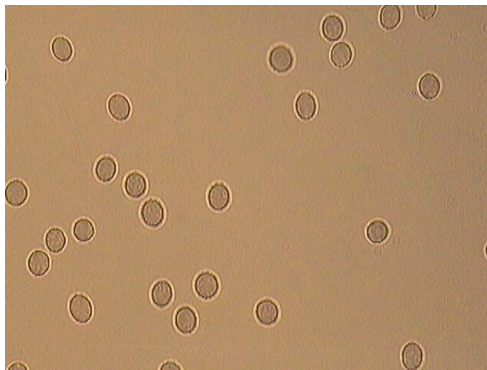


Figure 6 F4 and F5 formulations RBC.

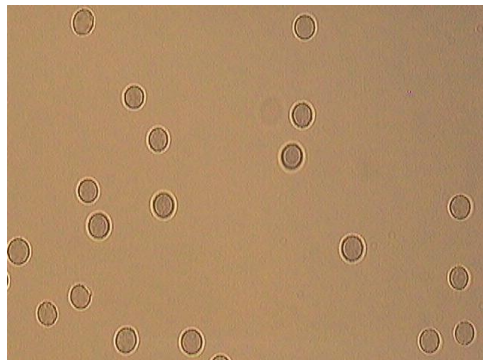


Figure 7 Innovator sample RBC.

CONCLUSION

- i) Egg Membrane after instilled the 0.9% sodium chloride (Negative control)
No haemorrhage,
No Vasoconstriction,
No lysis and
No coagulation.
- ii) Egg Membrane after instilled the Innovator sample :
No haemorrhage,
No Vasoconstriction,
No lysis and
No coagulation
- iii) Egg Membrane after instilled F5 formulation (Test product):
No haemorrhage,
No Vasoconstriction,
No lysis and
No coagulation
- iv) Egg Membrane after instilled 1.8% sodium chloride (positive control):
Haemorrhage observed
Coagulation observed

On the basis of above observation, our F5 formulation as test sample does not shows any abnormal effect Same observation on innovator and 0.9% sodium chloride (Negative control) , so F5 formulation is non-irritant hence our test product is safe for ophthalmic or ocular use

It can be also concluded that On the basis of above mentioned images of RBC in different solution, we concluded that the innovator sample showing similar i.e. isotonic image with normal RBC ,and in-house formulation (i.e F4,F5 and F6) showing does not any changes in the RBC's ie also similar to the innovator as well as normal RBC's.Hence on the basis of statement and images our formulation is isotonic, matching with innovator and our test product is safe for ophthalmic or ocular use.

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