



ISSN: 1697-090X

Inicio Home

Indice del  
volumen Volume  
index

Comité Editorial  
Editorial Board

Comité Científico  
Scientific  
Committee

Normas para los  
autores  
Instruction to  
Authors

Derechos de autor  
Copyright

Contacto/Contact:  


## Letters to the Editor / Cartas al Editor

# THE CHICK CHORIOALLANTOIC MEMBRANE AS A MODEL TISSUE FOR IRRITATION STUDIES ON MULTIPURPOSE SOLUTION

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### To the Editor:

The chick chorioallantoic membrane (CAM), a part of the extraembryonic tissue begins to develop 7 days after initial incubation from the fusion of the chorion and the allantois. Structurally, the outer epithelial layer of the chorion is derived from the trophoblast, which opposes the allantois. This structure forms a supportive matrix for the extensive vascular network that courses through the CAM, analogous to the retina and its vasculature. Overall mature chick CAM (20-100  $\mu\text{m}$ ) and human retina (approx 100-300  $\mu\text{m}$ ) are of roughly comparable thickness. Mature chick CAM (incubation day 12 and on) can be divided into three anatomically distinct layers. 1.- Primary stratum, 2.- Capillary plexus or blood sinus, 3.- A thin stratum.

The specialized chorionic epithelial cells that have presumably migrated above the capillary plexus are involved in gas exchange and calcium absorption. Immediately above and attached to CAM thin layer is the inner shell membrane<sup>1-3</sup>.

The irritation study was performed in order to ascertain the irritating effect if any of multipurpose solution (MPS) used for rinsing and soaking of hydrophilic contact lenses (CL's) before being placed into the eyes. In the present study CAM test was used for performing irritation studies on MPS used for hydrophilic CL's. The CL's are to be rinsed and soaked in multipurpose solution (MPS) before being worn. The composition of MPS coded as MPS-2 is given in table 1. In a fertile egg, the CAM test has been used extensively in the past for ocular angiogenesis studies. The tissue is a useful tool for ocular irritation studies including hyperaemia, hemorrhage and coagulation.

**Table-I Composition of MPS-2**

Ingredients	Qty.
Polyhexanide hydrochloride (20%)	0.0002%
Poloxamer 407	1.25%
Sodium citrate	0.1%
Disodium edetate	0.1%
HPMC 2906 grade	0.1%
PEG 400	0.1%
<b>Isotonic simulated tear fluid of pH 7.4...Q.S... (Prepared as per U.S.P.)</b>	<b>100 ml</b>

The CAM test is a convenient model as it involves low cost and hence considered less expensive than the living animal. The eggs are maintained at adequate humidity at  $37 \pm 0.5^\circ\text{C}$  temperature condition and rotated after every few hours. These conditions can be maintained in an incubator and the eggs of 14 days old are fit for experimental work. The CAM experiment can be performed between 12-17 days of incubation. Before 12 days, the CAM vasculature is not adequately matured and after 17 days the embryo is large enough and hence its size and movement underneath the CAM could disturb the experimental maneuvers on the CAM hence 12-14 days of incubation is the ideal time for experimental work<sup>4-8</sup>.

The test is based on the scoring scheme for hyperaemia, hemorrhage and coagulation parameters. Any increase in red color of CAM (hyperaemia) can be given a score number from 0 to 5. Similarly the haemorrhage (leaking of blood) can be given a score from 0 to 7 i.e. no haemorrhage at 0 and strong haemorrhage at 7. Scores from 0 to 9 is used for coagulation parameter. A detailed account is given in table 2.

**Table-II: Scoring scheme for hyperaemia, haemorrhage and coagulation.**

Effect	Scores
Hyperaemia (Any increase in the red colour of CAM)	0 - No hyperaemia
	1 - Very slight hyperaemia
	2 - Slight hyperaemia
	3 - Moderate hyperaemia
	4 - In between moderate and strong hyperaemia
	5 - Strong hyperaemia
Haemorrhage (Place filter paper on CAM, remove and see any blood content)	0 - No haemorrhage
	1 - Very slight haemorrhage
	2 - Slight haemorrhage
	3 - Slightly moderate haemorrhage
	4 - Moderate haemorrhage
	5 - Slightly strong haemorrhage
	6 - Moderately haemorrhage
	7 - Strong haemorrhage
Coagulation (Any change in colour of CAM rising depression in the membrane, granular or shaggy appearance of membrane or opaqueness of membrane)	0 - No coagulation
	1 - Obscure coagulation
	2 - Very light coagulation
	3 - Slight coagulation
	4 - Slightly moderate coagulation
	5 - Moderate coagulation
	6 - In between slightly strong and moderate coagulation
	7 - Slightly strong
	8 - Moderately strong
	9 - Strong

The cumulative scores were used for assessment of extent of irritation and taking hyperaemia, haemorrhage and coagulation into consideration as given below<sup>9-13</sup>. Cumulative scores and irritation assessment: 0 - 0.9 practically none; 1 - 4.9 slight; 5 - 8.9 moderate; 9 - 21.0 strong.

Multipurpose solution (MPS-2) prepared on pilot scale at Gaymed labs Delhi, India. Propylene glycol and Sodium lauryl sulphate from E Merck, Mumbai, India. Baby shampoo from Johnson & Johnson Mumbai. All other materials were used as received.

Fertilised chick eggs are easily ordered and procured from poultry farm and do not require an extensive animal protocol (as long as they are used and disposed before 19 days). These are considerably less expensive than the living animals currently used in ophthalmic and contact lens experimentation (e.g. rabbits, rats, cats, minipigs, dogs). Maintenance of eggs with 75% of relative humidity, a  $37^\circ\text{C}$  environment and rotation after 2 hours, can be performed in an inexpensive incubator.

Immediately after procurement the fertilized chick eggs (12 days old) were washed with water and kept on cotton surface already placed in a tray. The tray was kept in an incubator for 2 days at  $37 \pm 0.5$  °C. The eggs were rotated after every 2 hours (figure 1).



Figure 1.- One of the egg in the tray

To prepare CAM for typical experiment, the eggs were examined under candle light and only healthy and fertile eggs were selected. The egg shell is cracked and peeled away from the region over the air space that exists between the shell and the inner shell membrane (ISM) at one pole of the egg (figure 2).

This air space can be visualized before the egg is cracked by holding the egg under intense light. The eggs were cracked. The white part of the egg was removed carefully with the help of a pointed needle and a sharp blade in order to expose, once the opaque CAM-ISM dual layer is exposed, the irrigation with saline 0.9% will cause the dual layer to become translucent, allowing for visualization of the CAM vasculature as shown in (figure 3).



Figure 2.- The cracked egg shell.



Figure 3.- The CAM of Hen's fertile egg

The exposed CAM was divided into four parts 1, 2, 3 and 4. These were written on the four sides. The preparations were applied on these spots as a drop in the following manner: Area 1: Multipurpose solution coded as MPS-2. Area 2: Sodium lauryl sulphate (SLS). Area 3: Baby shampoo (Johnson). Area 4: Propylene glycol (PG)

Immediately, these were kept in the incubator and after 0.5, 2 and 5 minutes, the area were examined for hyperaemia, haemorrhage and coagulation. The resultant scores obtained were shown in table 3

**Table-III: Scores of Haemorrhage, Hyperaemia and Coagulation on CAM for MPS-2, Propylene glycol, SLS and Baby shampoo**

MPS - 2	Egg	Scores									Cumulative score for 5 min.
		Haemorrhage (A)			Hyperaemia (B)			Coagulation (C)			
		Time (in mins)			Time (in mins)			Time (in mins)			
		0.5	2	5	0.5	2	5	0.5	2	5	
Egg 1	0	0	0	0	0	0	0	0	1	A + B + C = 0.50	
Egg 2	0	0	0	0	0	0	0	0			
Egg 3	0	0	0	0	0	0	0	0	1		
Egg 4	0	0	0	0	0	0	0	0	1		
Egg 5	0	0	0	0	0	0	0	0	0		
Egg 6	0	0	0	0	0	0	0	0	0		
Mean	0	0	0	0	0	0	0	0	0.50		

Propylene glycol	Egg	Scores									Cumulative score for 5 min.
		Haemorrhage (A)			Hyperaemia (B)			Coagulation (C)			
		Time (in mins)			Time (in mins)			Time (in mins)			
		0.5	2	5	0.5	2	5	0.5	2	5	
Egg 1	0	0	0	0	0	0	0	0	0	A + B + C = 0.33	
Egg 2	0	0	0	0	0	0	0	0			
Egg 3	0	0	0	0	0	0	0	0	1		
Egg 4	0	0	0	0	0	0	0	0	1		
Egg 5	0	0	0	0	0	0	0	0	0		
Egg 6	0	0	0	0	0	0	0	0	0		
Mean	0	0	0	0	0	0	0	0	0.33		

Baby shampoo	Egg	Scores									Cumulative score for 5 min.
		Haemorrhage (A)			Hyperaemia (B)			Coagulation (C)			
		Time (in mins)			Time (in mins)			Time (in mins)			
		0.5	2	5	0.5	2	5	0.5	2	5	
Egg 1	0	0	0	0	0	1	0	0	1	A + B + C = 1.49	
Egg 2	0	0	0	0	0	1	0	0	1		
Egg 3	0	0	0	0	0	0	0	0	1		
Egg 4	0	0	0	0	0	1	0	0	0		
Egg 5	0	0	0	0	0	1	0	0	1		
Egg 6	0	0	0	0	0	1	0	0	0		
Mean	0	0	0	0	0	0.83	0	0	0.66		

Sodium lauryl sulphate	Egg	Scores									Cumulative score for 5 min.
		Haemorrhage (A)			Hyperaemia (B)			Coagulation (C)			
		Time (in mins)			Time (in mins)			Time (in mins)			
		0.5	2	5	0.5	2	5	0.5	2	5	
Egg 1	0	0	0	0	1	2	0	2	3	A + B + C = 10.99	
Egg 2	0	0	1	0	1	2	0	2	3		
Egg 3	0	0	1	0	1	2	0	3	5		
Egg 4	0	0	1	0	1	1	0	4	6		
Egg 5	0	0	1	0	1	2	0	2	6		
Egg 6	0	0	1	0	1	2	0	2	6		
Mean	0	0	0.83	0	1.00	1.83	0	2.50	4.83		

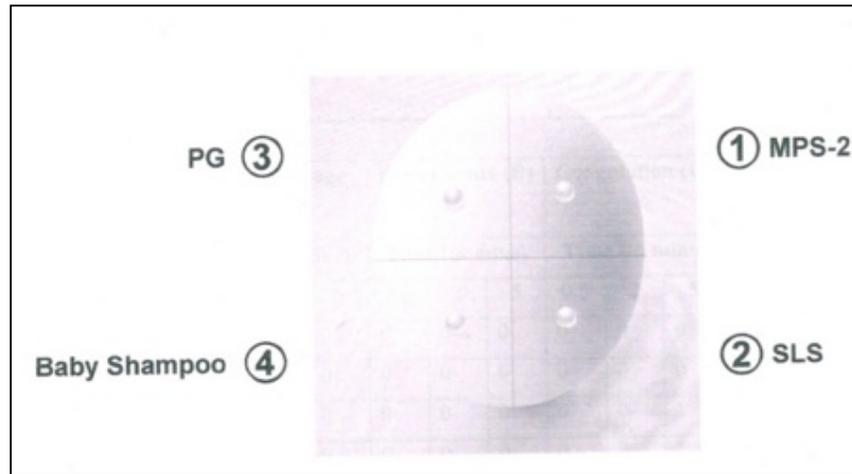


Figure 4.- Diagram Showing the Scheme of Application on CAM Sample.

The multipurpose solution coded as MPS-2 was formulated on pilot scale. The irritation studies on the preparation MPS-2 used as rinsing and soaking solution for hydrophilic contact lenses were performed using chorio allantoic membrane (CAM) test of fertile hen's egg. The preparation was applied as a drop on the CAM and scores for haemorrhage, hyperaemia and coagulation were observed and were recorded. The mean score was calculated and cumulative score was obtained. This was obtained as 0.50. As per the interpretation of standard parameter a cumulative score in between 0-0.9 is practically non-irritant. Therefore multipurpose solution MPS-2 was found to be practically non-irritant. For comparative purpose the test was also performed for propylene glycol, baby shampoo and sodium lauryl sulphate. For propylene glycol the cumulative score was 0.33 hence it is also nonirritant. This was practically observed on CAM. A substance is slightly irritant if the mean score is in the range of 1-4.9. For sodium lauryl sulphate, the cumulative score was 10.99 hence it indicated a strong irritation effect as it was in the cumulative score range of 9-21. The three materials that were used. Propylene glycol, baby shampoo and SLS as controls in the study.

Irritation studies should be carried out on preparation like multipurpose solution because it has to be used for rinsing and soaking of CL's (hydrophilic contact lenses). The CAM is very much similar to retina of the eye hence can be used for irritation studies on eye preparations. Such experiments are less expensive and do not require extensive animal protocol. Maintenance of eggs requires adequate humidity and a 37°C environment and rotation after every few hours, which can be performed in an inexpensive incubator.

The type of CAM experiments described herein can optimally be performed between incubation days 12 and 18. Before day 12, the CAM vasculature has not adequately matured. After day 18, the embryo is large enough that its size and movements underneath the CAM can disrupt experimental maneuvers on the CAM, Additionally, standard animal protocols for chick embryos past 18 require more complicated euthanasia techniques.

**Conclusion:** The optimized product MPS-2 and marketed by the name of Multisol is practically non irritant and can be used for rinsing, soaking, disinfecting, lubricating and deproteinising hydrophilic contact lenses.

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