Chapter 6
Irritation & Toxicity Studies

6.1 Introduction

Skin irritation is defined as a reversible inflammatory reaction produced by the arachidonic acid cascade and cytokines in the viable skin cells like keratinocytes and fibroblasts. Inflammatory cells, which are penetrated by the fibroblasts and blood vessels, also relate to skin irritation, resulting in redness and edema in the viable epidermis and dermis (Lawrence, 1997 and Nickoloff et al, 1994).

The manufacture, transport, and marketing of chemicals and finished products require the prior toxicologic evaluation and assessment of skin reactivity (corrosion and irritation) that may result from intended or accidental skin exposure. Traditionally, testing for skin corrosion and skin irritation has been conducted in animals (Organization for Economic Cooperation and Development, OECD, 1993).

In the mid 1940s, Draize published his method for assessing skin irritation hazard in rabbits (Draize et al, 1944). In essence, the method calls for the occluded application of test chemicals to rabbit skin for duration up to 24 h. To this day, different variations of this procedure form the basis for classification of skin corrosion and irritation hazard to man. Depending on the severity of the skin reactions (e.g., erythema, edema, necrotic changes), their speed of onset, and their persistence and reversibility, classifications of skin corrosion and irritation hazard are assigned.

A variety of cell-based biologic assay systems have also been developed over the past 10 years to investigate the dermatotoxic effects of chemicals and product formulations on the skin. These have included simple submerged cell cultures, submerged cell cocultures incorporating more than a single cell type.

Cell cytotoxicity assays are amongst the most common of the in vitro bioassay methods used to predict the toxicity of substances to various tissues. They have been used to examine organ-specific damage and the tissue inflammatory potential of chemicals. There are many cell cytotoxicity assays like MTT assay, Neutral red uptake assay (24 or 48 h exposure), Neutral red release assay (5 - 30 min exposure), Cell protein assay, Plasminogen activator assay, Red blood cell lysis assay etc. (Eun et al, 2000).

MTT assay

Tetrazolium dye MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide) and similar tetrazolium salts are converted from the oxidized to the reduced form by the NADH - dependent reaction catalyzed by succinate dehydrogenase. In its oxidized form MTT is yellow and it turns blue-black on reduction. The concentration of the reduced form is then measured spectrophotometrically.

Under stable conditions, the amount of MTT reduced per unit time is proportional to the cell number. It should be noted that this is also an indirect measure of cell viability and that test materials which increase mitochondrial activity also increase MTT reduction. MTT assay is prevalently used in various cell types and culture systems. It is one of the excellent methods of cell viability measurement using cell culture (Mosmann, 1983, Carmichael et al, 1987 and Ekwall et al, 1989).

Clinical Evaluations

4h Patch test

A human 4-h patch test has recently been developed and proposed as an alternative to the Draize rabbit test for assessing the irritation hazard potential of chemicals. It has been noted in multiple publications that the method is a

superior alternative to rabbit skin testing, since it involves testing of the relevant species (man), in addition to the relevant target organ (skin), and the appropriate clinical endpoint (skin irritation). The method relies on a simple statistical comparison of results for the test chemical with those of 20% sodium dodecyl sulfate, included in each study as a positive control (Robinson et al, 1998).

24-hour/48-hour occluded or semi-occluded patch tests

It is used for comparative assessments of one or more materials simultaneously in the same individual protocols, usually with diluted material. Test substances, diluted or undiluted, are usually applied to the skin of the upper arm or back for periods up to 48 hours. Attention has to be paid to the type of chamber or patch because of its influence on the result (Kim et al., 1987; York et al, 1995). Readings are performed at 1, 24 and 48 hours after removal of the patches. Parameters are visual effects such as erythema, dryness, oedema. Objective evaluations using observation of visual classification, TEWL and Laser Doppler flowmetry have been describe Reactions at the test sites should be scored throughout the test by the same experienced assessor who made the baseline assessment and under the same lighting source, following a pre-defined scoring scale (Walker et al, 1997).

Sub Acute Dermal Toxicity Study

In the assessment and evaluation of the toxic characteristics of a substance, determination of acute dermal toxicity is useful where exposure by the dermal route is likely. It provides information on health hazards likely to arise from a short-term exposure by the dermal route. Data from an acute dermal toxicity study may serve as a basis for classification and labelling. It is an initial step in establishing a dosage regimen in subchronic and other studies

and may provide information on dermal absorption and the mode of toxic action of a substance by this route.

The test substance is applied to the skin in graduated doses to several groups of experimental animals, one dose being used per group. Subsequently, observations of effects and deaths are made. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied. Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out.

6.2 Methods

6.2.1 Sub-Acute dermal toxicity testing

A sub-acute dermal toxicity study was carried out in wistar rats for duration for 2 weeks. The details of the procedure are given below:

Species: Wistar Rats

Number of animals in each group: 6 (3 males and 3 females)

Number of Groups: 4

Group Description and Dose level:

1) Placebo (PL)
2) Halobetasol propionate Microemulsion based cream 0.035% (HPMEC 0.035%)
3) Halobetasol propionate Microemulsion based cream 0.05% (HPMEC 0.05%)
4) Tacrolimus Microemulsion based cream 0.1% (TacMEC 0.1%)

Duration of the study: 14 days / 2 weeks

Application Regime: Every day for 2 weeks

Diet: Standard animal feed and water *ad libitum*

Environmental Conditions: Under Controlled temperature and humidity level

Procedure for the test: Healthy young adult animals were acclimatized to the laboratory conditions for at least 5 days prior to the test. Before the test, animals were randomized and assigned to the treatment groups. Approximately 24 hours before the test, fur was removed from the dorsal area of the trunk of the test animals by clipping or shaving. Care must be taken to

avoid abrading the skin, which could alter its permeability. Repeat clipping or shaving is usually needed at approximately weekly intervals. Not less than 10 per cent of the body surface area is made clear for the application of the test substance. The test substance was applied uniformly over an area which is approximately 10% of the total body surface area. During exposure the test substance is held in contact with the skin with a porous gauze dressing and non-irritating tape. At the end of the exposure period, residual test substance was removed using water and cotton. All the animals were observed daily and signs of toxicity recorded, including the time of onset, their degree and duration. Cageside observations include changes in skin and fur, eyes and mucous membranes. Measurements were made of food consumption weekly and the animals weighed weekly. The following examinations were customarily made on all animals including the controls:

(a) Haematology, including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leucocyte count, platelet count, was investigated at the end of the test period.

(b) Histopathological examination: Full histopathology was carried out on normal and treated skin in the control and high-dose groups.

(c) All gross lesions were examined.

Histopathological findings at Day 14:

The skin is excised and fixed in formalin and embedded in paraffin wax followed by section cutting and staining with hematoxylin eosin stain and toluidine blue (for mast cell specific). The cut sections are observed under microscope (10X) and photographic images are taken.

6.2.2 MTT assay

The extent of skin irritation of the mouse skin was evaluated by MTT conversion assay. The metabolic reduction of this soluble tetrazolium salt to a

blue formazan precipitate is dependent on the presence of viable cells with intact mitochondorial function. The MTT assays performed in this study essentially followed the procedure defined for monolayer cell cultures, modified to suit the mice skin. The hair from mice skin was shaved off 5 days prior to the test using an epilator. Microemulsion based creams of halobetasol propionate and tacrolimus were applied to the back of mice and secured using gauze pad. The application time was kept as 4h. After 4h, gauze pads were removed and mice were sacrificed immediately. 8 N potassium hydroxide was applied for 1 hour on excised skin as positive control and sterile distilled water as negative control. Then 1.2 ml of MTT solution (1.2 ml) at a concentration of 0.333 mg/ml in phosphate buffered saline (PBS) was applied on the dermis side. After reaction for 3 h at 37°C in a humidified atmosphere, the tissue sample was washed with 1.0 mL of PBS. A tissue disc having a diameter of 1.0 cm was made by biopsy punch. The obtained tissue was transferred to a test tube and was submerged in 1.0 mL of 0.04 N HCl in isopropanol. The formazan produced from the MTT conversion was extracted overnight at room temperature in the dark. The absorbance of the solution was measured at 570 nm using a spectrophotometer.

The optical density was used to calculate viability of the excised tissue to get a measure of the cytotoxicity potential of test substances. The relative tissue viability of each tissue was calculated as a percentage of the viability of the mean of the negative controls.

6.2.3 Evaluation of Skin Irritancy in Human Subjects

A 48-h human test for primary irritation was performed at Baroda Medical College, Vadodara. Twelve volunteers comprised the test panel. An occlusive patch bearing the test and comparative formulations was applied to the upper outer arm for an initial period of 23 h. One hour after patch removal, skin sites

were assessed for signs of skin irritation, which were rated with a numerical scoring system. Immediately after assessment, an identical fresh patch was applied to the same skin area for a further 23 h, and skin sites were again assessed 1 h after patch removal. The system of scoring took into account several different conditions of significance depending on the relative severity of the conditions. Each condition was scored as follows according to the strength of reaction observed:

Condition	**Rating**
Vesicles	5
Edema	4
Erythema	3
Flakiness	2
Dryness	1
Wrinkling	1
Glazing	1

In addition, each condition was scored according to the strength of observed reaction:

0 = no visible reaction;

1 = reaction just present;

2 = slight reaction;

3 = moderate reaction;

4 = severe reaction.

To obtain a numerical value for the total reaction at each site, the score for strength of reaction was multiplied by the corresponding condition rating, and the resulting values were summed to provide a global score for the

degree of irritation. For example, the total score for a site assessed as having slight erythema, moderate wrinkling, and severe dryness would be (2 x 3) + (3 x 1) + (4 x 1) = 13. The irritation scores for tested products were compared statistically with ANOVA to analyze both 24- and 48-h results.

Inclusion criteria:

- informed volunteers, where appropriate of relevant age, gender, ethnic origin and health condition
- panellists agree to follow the conditions specified in the Study Information Sheet

Exclusion criteria:

- pregnancy or nursing condition (except where specifically required)
- blemishes, marks (e.g. tattoos, scars, sunburn) on the test site(s) which could interfere with scoring
- medication that may affect skin response, or past medical history
- Irritated skin on test site(s)
- any active skin disease which may interfere with the aim(s) of the study
- participation in another simultaneous study
- Participation in a previous study without an appropriate rest period between studies.

6.3 Results

6.3.1 Sub-Acute Dermal Toxicity Study

Table 6.1: Observations of animals during the course of sub-acute dermal toxicity study

Group		Mean weight (g) ± SD*	Gross observation of animal	Any toxicity / death	Food & water consumption
Day 0	Placebo	270 ± 24	Healthy with no lesions	None	*Ad libitum*
	HPMEC 0.035%	266 ± 16	Healthy with no lesions	None	*Ad libitum*
	HPMEC 0.05%	275 ± 11	Healthy with no lesions	None	*Ad libitum*
	TacMEC 0.1%	270 ± 25	Healthy with no lesions	None	*Ad libitum*
Day 7	Placebo	269 ± 18	Healthy with no lesions	None	*Ad libitum*
	HPMEC 0.035%	265 ± 23	Healthy with no lesions	None	*Ad libitum*
	HPMEC 0.05%	271 ± 18	Healthy with no lesions	None	*Ad libitum*
	TacMEC 0.1%	262 ± 23	Healthy with no lesions	None	*Ad libitum*
Day 14	Placebo	268 ± 20	Healthy with no lesions	None	*Ad libitum*
	HPMEC 0.035%	266 ± 24	Healthy with no lesions	None	*Ad libitum*
	HPMEC 0.05%	268 ± 19	Healthy with no lesions	None	*Ad libitum*
	TacMEC 0.1%	264 ± 22	Healthy with no lesions	None	*Ad libitum*

*n=6

Table 6.2: Hematology Findings at Day 14

Values are mean ± SD for n=6

TEST	VALUE				Unit	Reference Range
	PL	HPMEC 0.035%	HPMEC 0.05%	TacMEC 0.1%		
Hemoglobin (Hb)	15.13 ± 1.17	14.80 ± 0.96	14.45 ± 1.06	14.98 ± 1.22	g/dl	10 - 16.7
Erythrocyte (RBC)	7.59 ± 0.28	7.34 ± 0.78	6.64 ± 0.52	7.07 ± 0.55	/cu. mm	4.60 – 9.19
Hematocrit (PCV)	42.17 ± 2.71	42.05 ± 4.18	41.72 ± 2.56	41.78 ± 2.97	%	34.0 – 53.0 x 10^6
MCV	55.65 ± 5.22	57.48 ± 5.99	62.85 ± 2.93	59.26 ± 5.74	fl	50.0 - 77.8
MCH	19.95 ± 1.92	20.20 ± 0.92	21.74 ± 0.15	21.23 ± 0.64	pg	16.0 - 23.1
MCHC	35.95 ± 3.3	35.38 ± 3.56	34.32 ± 1.18	35.90 ± 2.88	g/dl	28.2 - 34.1
Leucocyte – total	10250 ± 1288	10583 ± 1695	11133 ± 1326	11466 ± 2265	/cu. mm	6000 - 14000
Leucocyte differential						
Polymorphs	28.58 ± 5.31	27.9 ± 5.34	28.16 ± 3.86	28.17 ± 5.07	%	5.3 – 38.5
Lymphocytes	68.42 ± 7.64	67.98 ± 4.12	67.33 ± 3.61	72.00 ± 10.0	%	56.7 - 93.1
Eosionphils	1.83 ± 0.98	1.58 ± 0.49	1.83 ± 0.41	1.50 ± 1.049	%	0 – 3.4
Monocytes	2.83 ±1.83	2.5 ± 1.22	2.67 ± 1.21	3.33 ± 1.633	%	0 - 7.7
Platelet count	734500 ± 54298	687500 ± 47710	690166 ± 33801	715666 ± 57753	/cu. mm	6,85,000 – 14,36,000

Histopathology at Day 14

Placebo

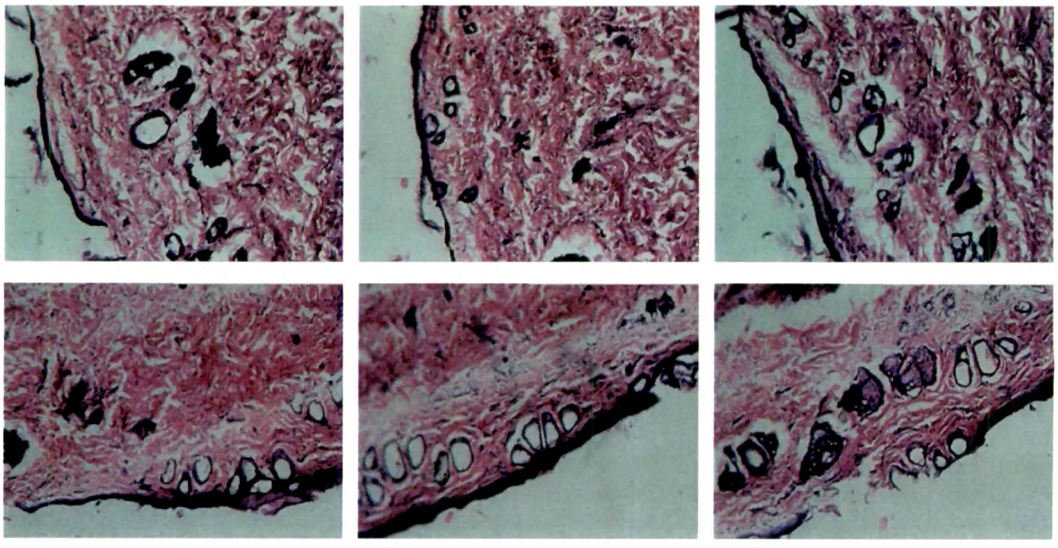

Fig 6.1: HE staining of rat biopsy for placebo group

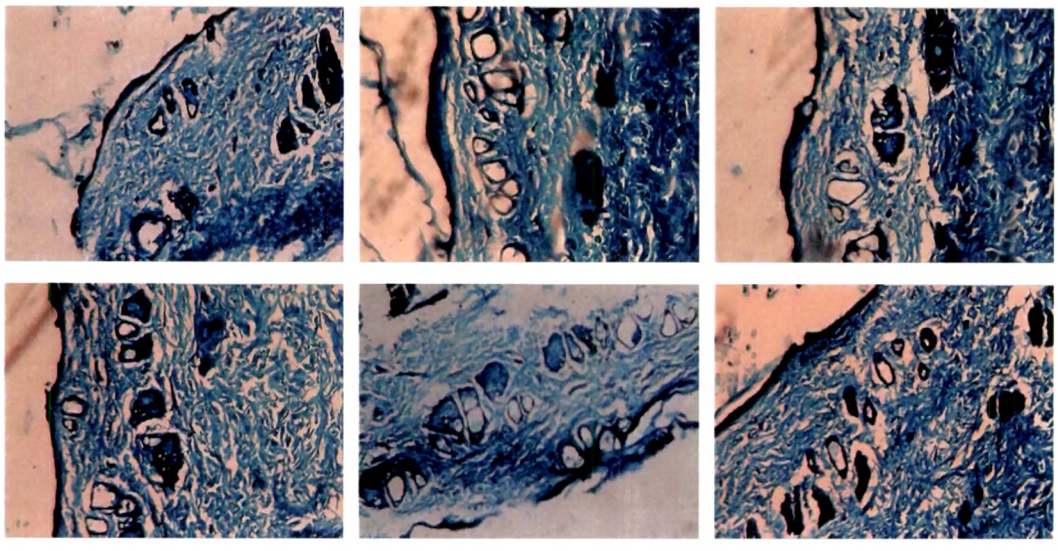

Fig. 6.2: Toluidine Blue staining of rat biopsy for placebo group.

HPMEC 0.035%

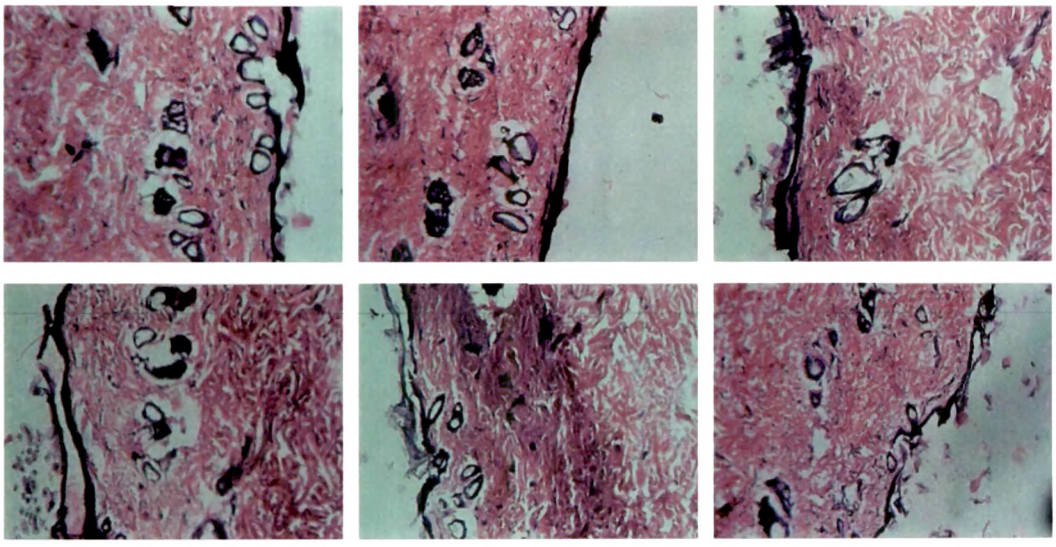

Fig. 6.3: HE staining of rat skin biopsy for HPMEC0.035% group

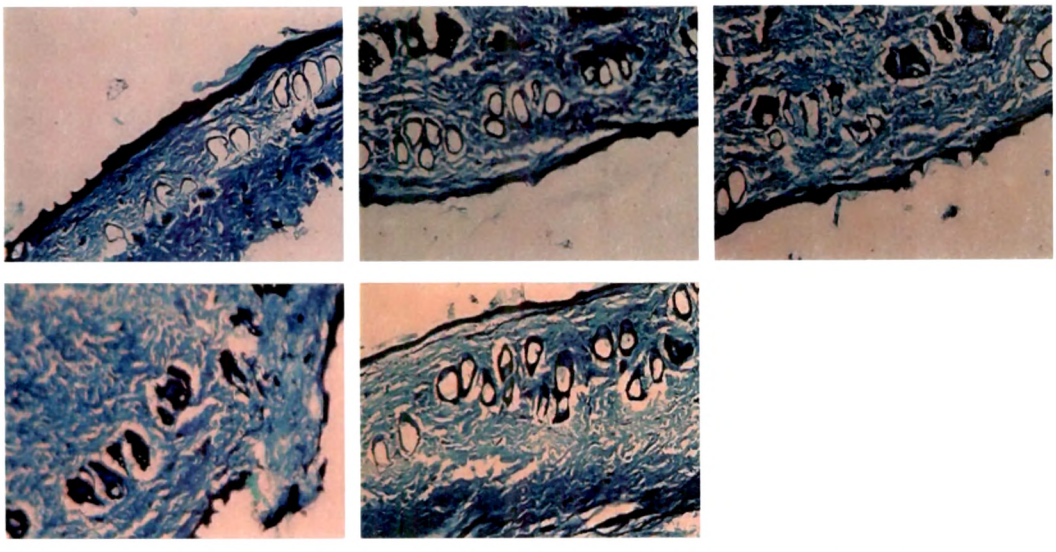

Fig. 6.4: Toluidine Blue staining of rat skin biopsy for HPMEC 0.035% group.

HPMEC 0.05%

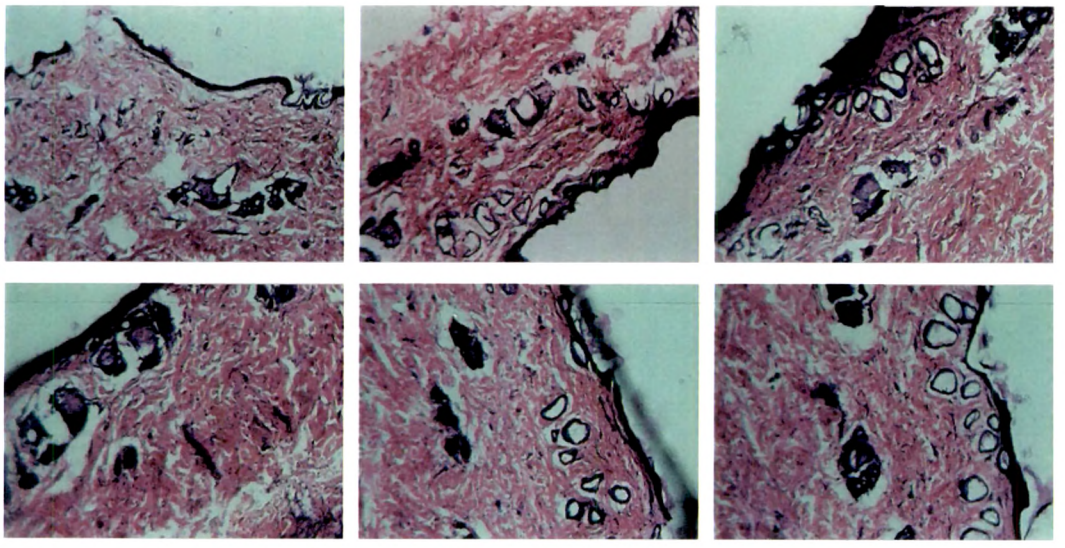

Fig. 6.5: HE staining of rat skin biopsy for HPMEC 0.05% group

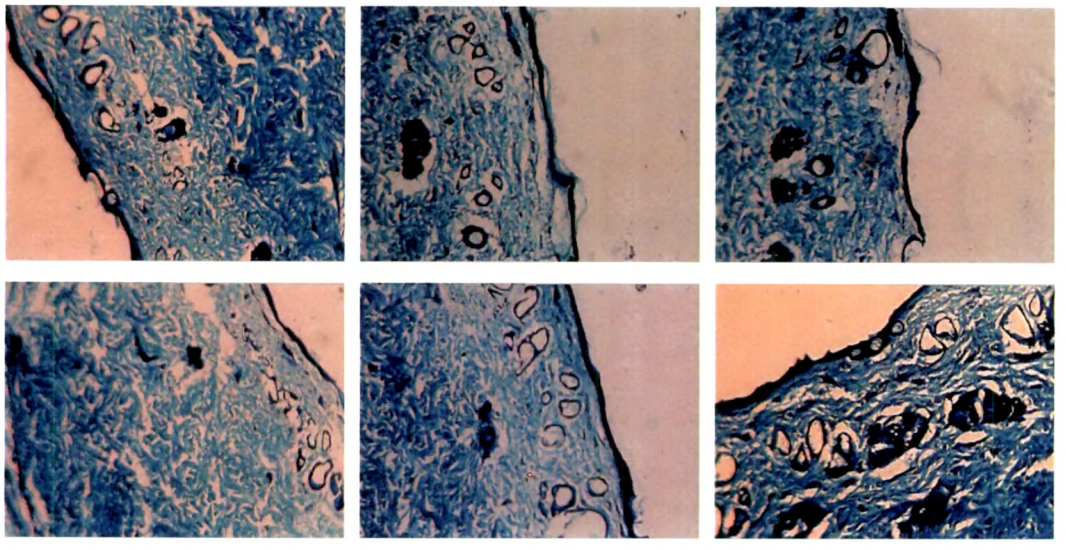

Fig 6.6: Toluidine Blue staining of rat skin biopsy for HPMEC 0.05% group.

TacMEC 0.1%

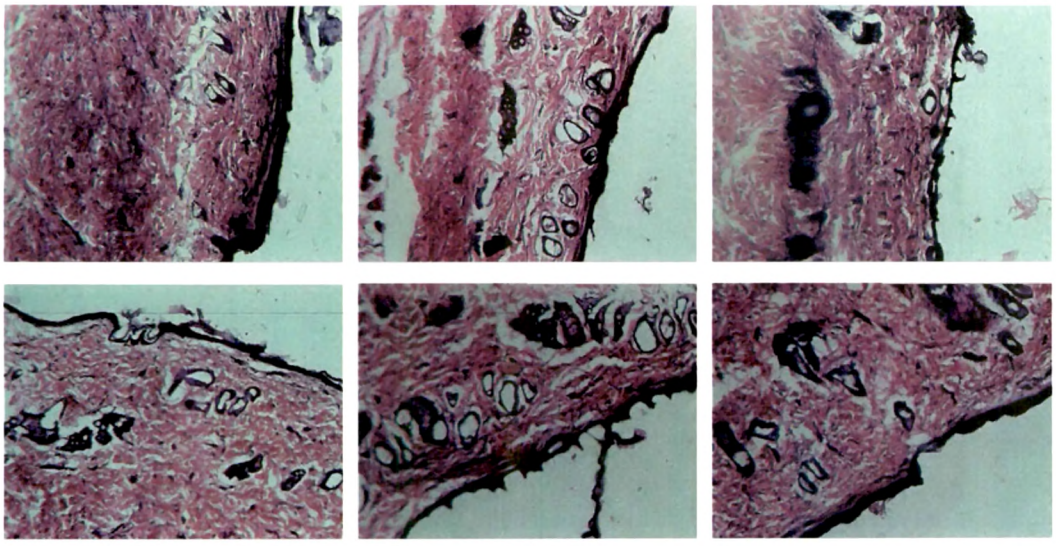

Fig. 6.7: HE staining of rat skin biopsy for TacMEC 0.1% group

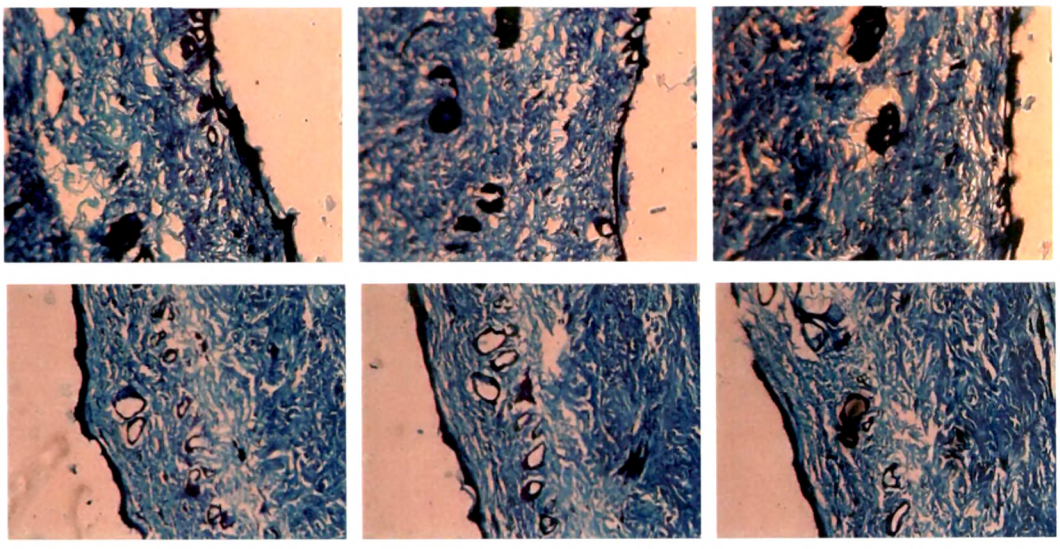

Fig. 6.8: Toluidine Blue staining of rat skin biopsy for TacMEC 0.1% group

Table 6.3: Number of mast cells

GROUP	PL	HPMEC 0.035%	HPMEC 0.05%	TacMEC 0.1%
n	6	6	6	6
Minimum	109.0	103.0	94.00	98.00
Maximum	142.0	123.0	143.0	142.0
Mean	125.5	115.7	113.7	120.8

6.3.2 MTT Assay

Table 6.4: Optical Density and relative viability by MTT assay

Test substance	Mean ± SD* (OD at 570 nm)	% Viability (relative)	Remarks
Sterile distilled water (negative control)	0.658 ± 0.078	100.0	-
8N KOH (positive control)	0.314 ± 0.038	47.72	Irritant
HPMEC 0.035%	0.597 ± 0.045	90.72	-
HPMEC 0.05%	0.568 ± 0.064	86.80	-
TacMEC 0.1%	0.613 ± 0.081	93.16	-

*n=3

6.3.3 Human Acceptance Testing

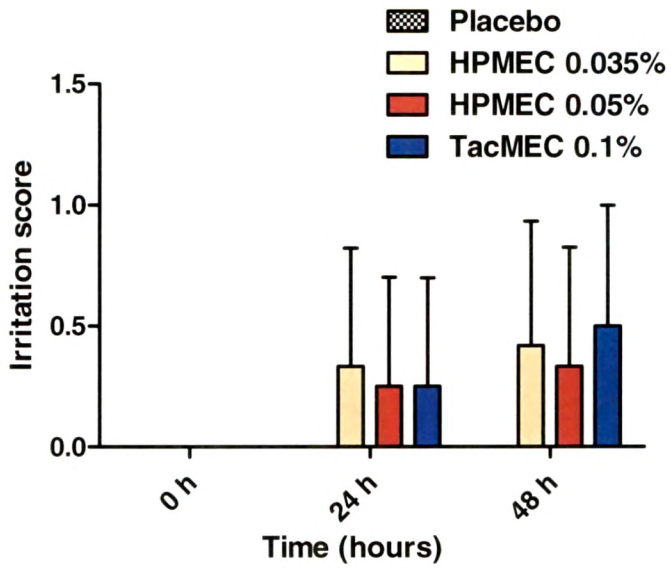

Fig 6.9: Irritation score in human subjects over a period of 48 hours.
HPMEC: Halobetasol propionate microemulsion based cream
TacMEC: Tacrolimus microemulsion based cream

6.4 Discussion

A sub-acute toxicity study of the formulations was carried out on wistar rats for duration for 2 weeks. The animals were divided into 4 groups each containing six animals, (3 males and 3 females) viz. control (Placebo), HPMEC 0.035% and 0.05% and TacMEC 0.1%. Daily gross observations of the animals and their food consumption were made. Their body weights were periodically measured. At the end of 2 week study, blood was removed from the animals and they were sacrificed and the excised skin from the site of application was removed and histopathology was performed. The blood indices were measured on a fully auto counter. It was observed during the study that the animals did not exhibit any signs of local toxicity (edema, erythema or eschar formation) at the site of application in all the four groups. The body weight trends also did not document any significant change of body weights of the animals during the study. Their food and water intake was not affected adversely in any of the groups. The blood indices like erythrocyte count, PCV, MCH, MCHC, Leucocyte counts (total and differential) did not exhibit any significant difference. The Hematoxylin–eosin stain of the sections of the excised tissue also did not demonstrate any epidermal toxicity or subepidermal signs of inflammation, edema etc. for all the 4 groups. Toluidine blue stain was used for calculating the number of mast cells. Mast cells are indicative of any allergic or immunologic response in the skin in response to application of the cream. The number of mast cells also reflected the same trend, with no significant difference between any of the groups.

The extent of skin irritation of the mouse skin was evaluated by MTT conversion assay on excised tissue. The metabolic reduction of this soluble tetrazolium salt to a blue formazan precipitate is dependent on the presence of viable cells with intact mitochondorial function. It can be seen from the

optical density and relative viability data (table 6.4) that all the developed formulations are relatively non toxic and non-irritant. The tissue undergoes severe necrosis when treated with positive control and relative viability reduces drastically (Watanabe et al, 2002).

Acceptability testing in humans under the conditions of this test, the placebo cream scored lower than HPMEC 0.035%, HPMEC 0.05% and TacMEC 0.1%. However, there was no statistical significance indicating that there was no significant difference between the four products tested at either assessment at 24 h and 48 h. Hence, the product may be regarded as non-irritant for human use (Friedman et al, 1995).

6.5 References

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