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Chick Chorioallantoic Membrane as an In Situ Biological Membrane for Pharmaceutical Formulation Development: A Review

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The use of animals in research has always been a debatable issue. Over the past few decades, efforts have been made to reduce, replace, and refine experiments for ethical use of experimental animals. The use of chick chorioallantoic membrane (CAM) was one of the proposed alternatives to the Draize rabbit ocular irritation test with several advantages including simplicity, rapidity, sensitivity, ease of performance, and cost-effectiveness. The recent use of CAM in the development of pharmaceuticals and testing models to mimic human tissue, including drug transport across CAM, will be discussed in this review.

Keywords Draize test; chick chorioallantoic membrane; formulation development; drug delivery; transport study; permeation

INTRODUCTION

The use of animals in research has always been a sensitive, sometimes contentious issue. Over the past few decades, with the push to meet the "3R" concepts defined by Russell and Burch in 1959, scientists have made attempts to reduce, replace, and refine experiments for the ethical use of experimental animals (Kolar, 2006; Russell & Burch, 1959). Besides having to adhere to the guidelines established by their institution's Institutional Animal Care and Use Committee (IACUC) on the use and care of animals for research purposes, researchers now have to provide clear justifications for the need to use animals for experimentation in grant applications, even before starting on the actual research work (Wigglesworth, 2006). In the cosmetic industry, efforts had been made to find alternatives to the Draize test (Draize, 1944), which was the standard procedure for assessing eye irritancy potential of a chemical. From these endeavors, the use of chick chorioallantoic membrane (CAM) was developed and validated as an alternative to the Draize test for eye irritancy testing (Leighton et al., 1985).

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The Fund for the Replacement of Animals in Medical Experiments (FRAME) and its Alternatives Laboratories (FAL) have carried out significant in vitro tests with the aim to replace existing in vivo toxicity tests (Clothier, Atkinson, Garle, Ward, & Willshaw, 1995). The time of exposure of the test substances on the CAM ranged from 0.5 min (Luepke, 1985) to 48 h (Lawrence, Groom, Ackroyd, & Parish, 1986). Different endpoints/scoring systems were developed to assess the effects of the test materials, such as (a) macroscopic assessment, including number of embryo deaths, size of reaction, and appearance of CAM (Lawrence et al., 1986; Leighton et al., 1985); (b) microscopic assessment (Lawrence et al., 1986); (c) cumulative score (Luepke, 1985); (d) statistical analysis and calculation (Brantner et al., 2002); and (e) a combination of any of these methods (Saw, Heng, Chin, Soo, & Olivo, 2006; Saw, Olivo, Chin, Soo, & Heng, 2005). Various test substances have been employed on CAM and these include (a) chemicals such as ethanol, Tween 80, sodium hydroxide, propylene glycols, and polyethylene glycols; (b) detergents; (c) cosmetics; (d) drugs, and (e) drug formulations. The formulation aspect will be the focus of this review. Attempts have also been made to relate CAM assays with other test methods employed (Martins, Pauluhn, & Machemer, 1992).

The CAM model is a promising substitute, likened to an intermediate in vivo system—one that is in between in vitro cell culture systems and in vivo animal models. The CAM is sensitive and responds relatively rapidly to chemical or biological actions of testing materials and drugs. Compared with animal models, chicken eggs are relatively inexpensive and easier to handle for experimentation. The use of chick embryo in in vivo experiments has also gained better social acceptance. Moreover, chick embryo studies done in the earlier stages of development are subjected to simpler protocols. Meanwhile, the Draize test has also received much criticism; some reasons of which are the difficulty in reproducing the findings, subjective scoring and interpretation, use of high dose of test material, results being "over-predictive" of the human response, and pain experienced by the animals during the test procedure

(Anderson & Russell, 1995). From a literature search on very recent reports on formulation development, formulation studies appear to still rely largely on the Draize rabbit test and also other animal corneas for evaluating cutaneous irritation and ocular tolerance (Lallemand et al., 2005; Pawar & Majumdar, 2006). Nevertheless, there have been several recent reports on the use of CAM for assessing the effects of pharmaceuticals, e.g., in formulations studies and drug screening (Table 1). The CAM can be used to develop pharmaceutical formulations of various drugs, including preformulation screenings for toxicity and efficacy of delivery systems (Alany, Rades, Nicoll, Tucker, & Davies, 2006; Chin, Lau, Bhuvaneswari, Heng, & Olivo, 2007; Lange, Ballini, Wagnieres, & van den Bergh, 2001; Pegaz et al., 2006; Saw et al., 2005, 2006; Vargas et al., 2004; Winternitz, Jackson, Oktaba, & Burt, 1996), testing of biologically active drugs (Ribatti, Nico, Morbidelli et al., 2001), investigations on the effects of hydrophilicity and lipophilicity of drugs (Ruck et al., 1998), and drug transport studies (Saw et al., 2005).

From a detailed search performed on available literature, it is found that the use of CAM for pharmaceutical formulation development has not been reviewed. Therefore, this mini review will focus on the use of CAM as an in vivo biological membrane for pharmaceutical formulation development. Researchers from both the pharmaceutical industry and academia will benefit from the use of the relatively cheap, simple yet sensitive CAM models. In comparison, animals models are more expensive, tedious, and also subjected to tighter regulations. Recent research publications highlighting the use of CAM as the alternative/potential models (Table 2) for different human tissues, such as the skin and eye, and on the use of CAM for transport studies will also be discussed briefly.

USE OF CAM IN PHARMACEUTICAL PRODUCT DEVELOPMENT

CAM is the major respiratory organ of the chick embryo that is formed by the fusion of three layers: chorionic ectoderm, allantoic endoderm, and mesoderm (containing blood vessels) (Mysliveckova & Rychter, 1975) (Figure 1). The highly vascularized CAM is a well-known model system especially in angiogenesis/antiangiogenesis research with several reviews published (Ribatti, Nico, Vacca et al., 2001; Richardson & Singh, 2003). In 1984, the anatomy of CAM was studied using scanning electron microscopy (McCormick, Nassauer, Bielunas, & Leighton, 1984). The report provided useful information for a better understanding of the physiology of CAM and relevant anatomical information relating to the use of CAM as an alternative to the Draize rabbit eye irritancy test. CAM is used in toxicological and tumor cell assays as human mimicry tissues, such as the eye and skin, and in studies that simulate the tissue repair process (Ribatti et al., 2003). As CAM is immunodeficient, different cells including human carcinoma cells have been successfully grown on CAM for various experimental studies (Table 2).

Photosensitizers are drugs that can be activated by a specific wavelength of light for the diagnosis or treatment of cancer, commonly referred to as photodynamic diagnosis (PDD) or photodynamic therapy (PDT), respectively. The major mechanisms of cancer PDT are through direct cell damage by generation of free radicals and secondary damage to the tumor vasculature supplying nutrients and oxygen for tumor growth. Over the past decade, photosensitizers have been the major class of pharmaceuticals researched on and many studies adopted CAM models. Besides testing therapeutic efficacy, the highly vascularized and transparent CAM allows in vivo examination and monitoring of treatment effects. As CAM has a certain degree of similarity to human eye and skin tissues, it can also be used as a model to screen chemicals or drugs for potential phototoxic effects. The CAM is an acceptable conjunctival model. Screening of phototoxic potential is important in the safety assessment of drugs at preclinical stage. Besides drug screening, drug formulations/dosage forms can be evaluated and compared using CAM models (Table 1).

Hidalgo (2001) reported on the various limitations inherent in conventional transport studies with in vitro artificial membranes. For example, the tendency of poorly soluble compounds to undergo extensive nonspecific surface binding in the experimental setup complicates the evaluation of the permeability rates of test compounds. Recently, Saw et al. (2005) successfully demonstrated that the CAM can be used as an alternative in vivo model for transport studies. Thus, CAM has the potential for overcoming limitations associated with artificial membranes. With proper measurements of the thickness and area of CAM, the amount of drug transported across CAM can be determined (Figure 1). This makes CAM an attractive in vivo model to screen different effects of drugs and permeation properties of penetration enhancers on drug delivery across CAM.

PREPARATION OF CAM

Egg embryo had been used for scientific experimentation since almost a hundred years ago for the implantation of tumor cells (Rous & Murphy, 1911). The living CAM was first developed for the culture and assay of viruses (Beveridge & Burnet, 1946) and was later adapted for other investigations.

Preparation of the CAM for experimentation should be carried out in the tissue culture hood to avoid problems relating to contamination (Saw et al., 2005; 2006). There are three more commonly reported methods in the literature to gain access to CAM: (a) dropped membrane technique (in ovo), (b) Zwilling technique (in ovo), and (c) shell-less technique (ex ovo). The choice of the method depends on the experimental design, requirement for assessment, and age of the embryo. Every method has its advantages and limitations, and they are

TABLE 1
CAM in Pharmaceutical Development/Drug Screening

Mode of Administration	Drug/Formulation/Dosage Form	Research	Reference
Intravenous	Porphyrin, meso-tetra(*p*-hydroxyphenyl)porphyrin (*p*-THPP), into polymeric biodegradable poly (D,L-lactide-co-glycolide)/nanoparticles	PDT studies	Vargas, Eid, Fanchaouy, Gurny, & Delie, 2008
Topical	Hypericin-*N*-methyl pyrrolidone and hypericin-human serum albumin/neat solution	PDD, preclinical studies of hypericin in fluorescence detection for bladder cancer implanted in CAM	Saw, Olivo, Chin, Soo, & Heng, 2007
Topical	Meso-tetra(hydroxyphenyl)chlorin (*m*-THPC) including conventional or plain liposomes (Foslip) based on dipalmitoylphosphatidylcholine (DPPC) and the corresponding long-circulating poly(ethylene glycol) (PEG)-modified liposomes (PEGylated liposomes: Fospeg)	PDT, preclinical studies of photothrombic activity	Pegaz et al., 2006
Topical	Water-in-oil microemulsions	Studies on ocular irritation potential of components and formulations	Alany et al., 2006
Topical	Chlorin e6-polyvinylpyrrolidone/neat solution	PDD and PDT studies	Chin et al., 2007
Topical	Hypericin-*N*-methyl pyrrolidone/neat solution	PDT antivascular effects, formulation screening, and hypericin transport studies	Saw et al., 2005, 2006
Topical	Liposomal and aqueous formulations of povidone-iodine	Angioirritative potential on CAM	Wutzler, Sauerbrei, Härtl, & Reimer, 2003

Topical	Photofrin, 5-aminolevulinic acid (ALA), benzoporphyrin derivative monoacid ring A (BPD-MA) and lutetium texaphyrin (Lutex)/neat solution	Studies on PDT parameters	Hammer-Wilson, Cao, Kimel, & Berns, 2002
Topical	Recombinant fibroblast growth factor-2 (FGF-2)-methylcellulose/dried disc with formulation	Studies on angiogenesis, wound healing process	Ribatti et al., 1999
Topical	Fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF)/gelatin sponges	Angiogenesis studies	Ribatti, Nico, Morbidelli et al., 2001
Topical	Taxol-poly(epsilon-caprolactone) (PCL) or blends of PCL with methoxypolyethylene glycol, molecular weight 350 (MePEG)/surgical paste	Anticancer chemotherapy studies	Winternitz et al, 1996
Intravenous/ topical	Intravenous hydrophilic tetrasulfonated aluminum phthalocyanine ($AlPcS_4$) and lipophilic liposomal zinc phthalocyanine (ZnPc), topical 5-aminolevulinic acid (5-ALA)	Drug distribution studies on CAM	Ruck et al., 1998
Intravenous	Porphyrin, meso-tetra(*p*-hydroxyphenyl)porphyrin (*p*-THPP), into polymeric biodegradable poly(D,L-lactide-co-glycolide)/ nanoparticles	PDT and solvent screening studies	Vargas et al., 2004
Intravenous	Liposome-encapsulated benzoporphyrin derivative monoacid ring A (BPD-MA, verteporfin)	PDT, age-related macular degeneration	Lange et al., 2001
Intraperitoneal	Photofrin-based 5-aminolevulinic acid (ALA), sulfonated chloro-aluminum phthalocyanine (AlPcSn), benzoporphyrin derivative monoacid ring A (BPD-MA), and lutetium texaphyrin (Lutex)/neat solution	PDT parameters studies	Hammer-Wilson, Akian, Espinoza, Kimel, & Berns, 1999

TABLE 2
CAM as Alternative to Human Cells/Tissues

Proposed Mimicry Tissue—Experimental System	Reference
Eye—surgical retinal research and simulation	Leng et al., 2004
Skin–short-term in vivo system	Kunzi-Rapp, Ruck, & Kaufmann, 1999
Skin—model for drug phototoxicity studies	Neumann et al., 1997
Bladder—MGH human bladder carcinoma cells implanted as a tumor model	Saw et al., 2007; Chin et al., 2007
Prostate—LNCaP, PC-3, and Tsu-Pr1 human prostatic cancer cell lines as well as 2 immortalized normal human prostate epithelial cell lines inoculated on CAM model—Early in vivo cancer research test system	Kunzi-Rapp et al., 2001

presented in Table 3. The day when an egg is placed in the incubator is designated as day 1 of the incubation or embryo age 1 (EA 1) (Lawrence et al., 1986). Eggs undergoing hatching are turned by a rotator to prevent the embryo from sticking to one side of the egg shell until a procedure is carried out to gain access to the CAM. Once the egg shell is broken, the egg is kept stationary.

The freshly dropped membrane method prepares the "classical" artificial air sac and is the most commonly used method. This method of preparation is described as follows: Fertilized eggs are first disinfected with 70% alcohol before placing them in the egg incubator at 37°C with 60% humidity. At EA 6–7 days, a window opening is punctured at the blunt end of the egg facing upwards using sterilized forceps. The opening is then covered with parafilm to avoid contamination and the egg is returned to the incubator for further incubation till EA 12–13 when the vessels are matured. The CAM has to be sufficiently matured before it is ready for experimentation. The reasons for using EA12 CAM are discussed in detail later as an approach to minimize variations, especially for experiments that evaluate the changes in sizes of vessels before and after treatment.

As mentioned earlier, requirement for assessment is one of the factors to be considered in method selection. The type and complexity of CAM depend on the development stage of the embryo. At EA 10, the three-dimensional development of the CAM is complete and the CAM attains maximum size (Mysliveckova & Rychter, 1975). CAM earlier than EA 12 may be used when only a macroscopic assessment of the effects of the test materials on CAM is needed without quantitative assessment.

The Zwilling technique does not involve dropping the membrane and was developed to overcome the traumatic damage to the membrane experienced in dropped membrane method (Zwilling, 1959). A window is made at the pointed end of the egg on EA 3, before the CAM is fully formed, to drain off 1.5–2 mL of albumin. After draining, the hole is sealed as the residue albumin dries across the opening. The removal of the albumin provides an air sac just under the shell and protects the organism from damage during subsequent manipulations. A second window will be created for access to the CAM at the equator of the egg for experiments to be carried out few days later.

Both dropped membrane and Zwilling methods involve conducting experiments with intact CAM inside the shell. A shell-less method was developed more recently and it involves pouring out the egg content on EA 3 into a petri dish. The dish acts as the shell and this preparation is particularly useful for angiogenesis research when evaluating anticancer drugs and ocular irritation tests (Alany et al., 2006).

Drugs either as neat solutions or in dosage forms could be applied directly to the CAM topically, intravenously, or intraperitoneally (Figure 1). Various formulations tested on CAM include solutions, liposomes, and nanoparticles (Table 1).

VARIABLES TO BE CONSIDERED FOR QUANTITATIVE AND QUALITATIVE EVALUATION OF CAM

The difficulties in quantifying responses on CAM have been well recognized (Richardson & Singh, 2003). It was criticized that the morphological evaluations based on responses were very cumbersome and provided results that were, at best, semi-quantitative (Maragoudakis, Panoutsacopoulou, & Sarmonika, 1988). Nonetheless, efforts were directed at improving the assessment system by various techniques (Saw et al., 2005; 2006).

Variables that can affect the assessment of responses on CAM are primarily of biological origins: (a) age of the embryo; (b) confounding effects of the drug delivery system on CAM tissues and vessels; (c) any nonspecific inflammation caused by additives added that is not apparent when observed visually; and (d) methodology for evaluating effects on CAM tissues and vessels (Saw et al., 2005).

APPROACHES TO MINIMIZE VARIATIONS IN ASSESSMENTS USING CAM

The embryonic age of CAM is an important variable to be considered. It is best to use mature CAMs of EA 12 and older as reduced biological variations are found. The vascular density of CAM had been reported to increase by more than three-fold from EA 8 to 12 and the rate of neovascularization was observed to be at its maximum between EA 10 and 11 (Maragoudakis et al., 1988). The vascular density reached a plateau by EA 12 and thereafter. Before EA 11, the vascular endothelial cells of CAM were still immature and at a high rate of cell division. It was postulated that these immature cells may affect the ability of the CAM to support implanted tissues/

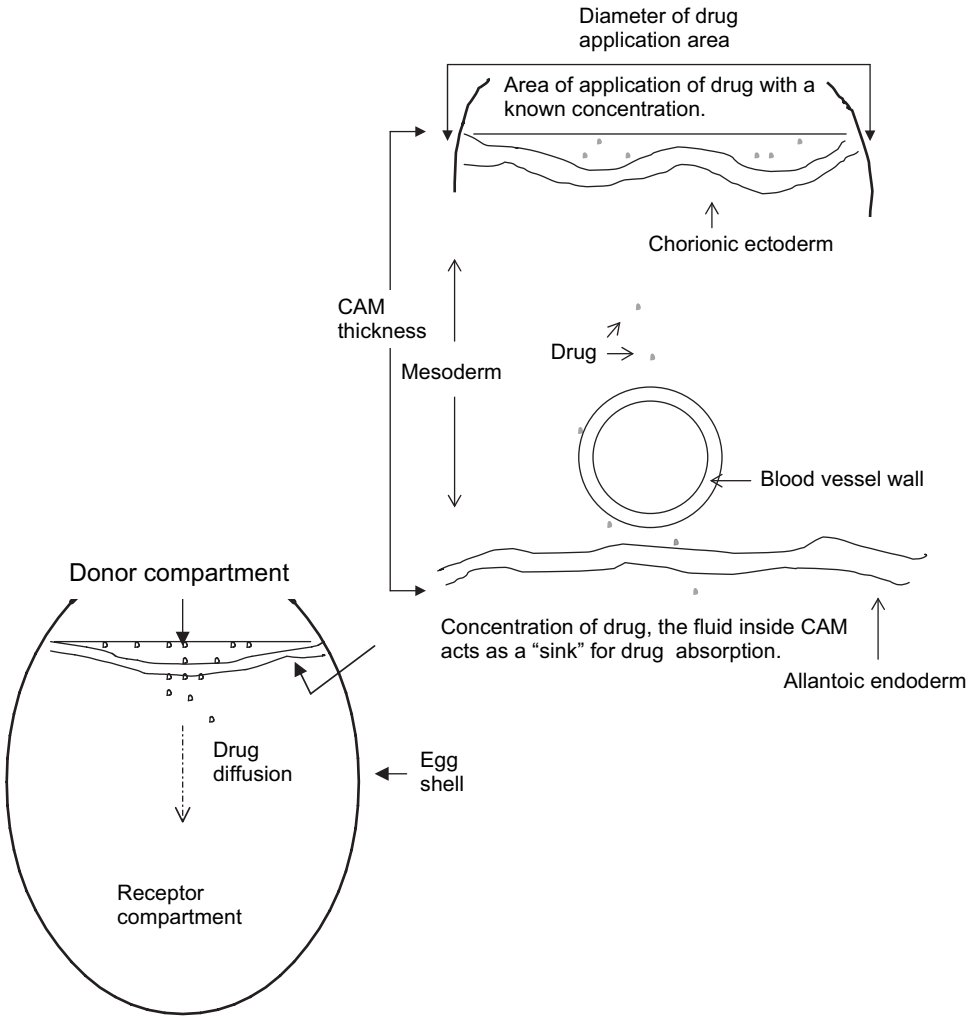

FIGURE 1. Schematic representation of a cross-sectional view of the chorioallantoic membrane (CAM) model for drug transport studies. Blow-up diagram indicating one underlying blood vessel (not drawn to scale) showing the components of CAM and drug diffusion through the CAM.

cells (Ausprunk, Knighton, & Folkman, 1974). If studies were to be carried out on CAM although vessels are still actively forming (before EA 12), vessel growth and the immaturity of the CAM itself (Romanoff & Romanoff, 1967) may be confounding factors in the quantification of the effect of drug dose (measured by the strength of an observed effect).

A proper experimental design with appropriate controls is needed for validated measurements. For example, a drug-free formulation to serve as negative control should be included in the experiment to rule out the false-positive effects brought about by the additives used in the formulations (Table 4). Likewise, in certain experiments, positive controls with known amount of a drug, be it the actual drug or another bioactive compound, may be needed.

Standardized image-capturing procedure and image analysis can reduce and limit variations by subsequent morphometric assessments of CAM responses. An example of morphometric assessment is the measurement of changes on vessel diameters as a result of drug/formulation effects. For the image-capturing procedure, variations in measurements can result from the distance between CAM and the camera lens, the position of the egg, and the magnification factors. Saw et al. (2005) used a standardized imaging procedure for image capture and allowed subsequent image analysis of the vasculature damage to be comparable. The camera, stereomicroscope, and magnification used must be fixed to provide consistent image capture (Figure 2). In addition, images of the CAM surface can be taken at various locations, for example, upper left quadrant, upper right quadrant, lower left quadrant, lower right quadrant, and central area to cover the whole surface of CAM.

Baseline measurements of vessels on CAM are necessary before, after, and during the experiments (Table 4). Inter and

TABLE 3
Comparisons of Dropped Membrane, Zwilling and Shell-Less Techniques

Technique	Dropped Membrane	Zwilling	Shell-Less
Location of embryo	In ovo	In ovo	Ex ovo
Embryo growth	Normal development of embryo in its natural host	Normal development of embryo in its natural host	Artificial host for embryo
Simplicity of procedure	The simplest procedure, only one window is created	Two windows are created; higher contamination risk and trauma to embryo	A more complex procedure that requires sterility to be maintained throughout the whole procedure till completion of experiments. The embryo is transferred to petri dish before the CAM is fully developed and further incubated outside the eggshell
Major potential problem	CAM is easily damaged during the preparation when piercing of the shell membrane that is in close contact with the CAM. Particles of shell falling onto the CAM during drilling	Risk of perforating egg membrane	Highest risk of contamination
CAM area	Small	Small	Large
Areas of application	For angiogenesis, antiangiogenesis, irritancy and screening of drugs, and formulations, including in situ transport studies	For angiogenesis, antiangiogenesis, irritancy and screening of drugs, and formulations, including in situ transport studies	For angiogenesis, antiangiogenesis, irritancy and screening of drugs, and formulations, but difficult for in situ transport studies

TABLE 4
Intra- and Inter-Groups Comparison for Quantification of Vessel Diameter

	Control Group	Experimental Group
Before experiment	*a*	*b*
After experiment	*c*	*d*
Intra-group comparison: effect size of each group	$\|c–a\|$	$\|d–b\|$
Inter-group comparison: establishment of baselines	$\|a–b\| = 0$ or close to 0, $p > .05$	
Inter-group comparison: quantification for size effects of drugs	$\|c–a\|–\|d–b\| \neq 0$, $p < .05$	

a and *b* represent the median of CAM vessel diameters for control and experimental groups before experiment. Please refer to the text for further details and interpretation of this table.

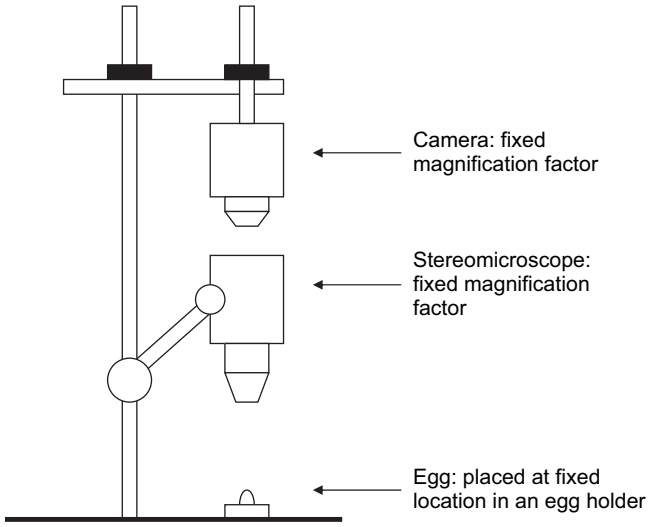

FIGURE 2. The experimental set-up of camera and stereomicroscope for the standardized image-capturing procedure. The magnifications of the camera and the stereomicroscope should be fixed at all times.

intra CAM blood vessel measurements before and after experiments are also required. Quantification and analysis can be made by nonparametric statistics as CAM vessels are not normally distributed. With careful reference baseline measurements, quantitative assessment of responses becomes possible (Saw et al., 2005). Before the start of the actual experiment, the vessel diameters on CAM should be determined across different vessel groups (a, control group; b, experimental group in Table 4) to obtain valid baseline measurements. The effect of drugs/formulations can be compared only when all experimental groups have similar baseline values (the absolute value of the difference between a and b is zero or close to zero, and the *p* value for the control and experimental groups is >.05, i.e., non-significant difference between the groups, Table 4). Nonparametric statistical analysis on measurements before treatment between control and experimental groups should show non-significant differences to establish an acceptable baseline. This allows for subsequent size effects comparison after measurements. After administration of drugs/formulations, the quantifiable effects brought about by addition of drugs/formulations can be obtained by measuring changes in vessel diameters (c, control group; d, experimental group in Table 4). It can then be concluded that the drugs/formulations have an effect on the CAM vessels when the absolute value of the difference between c and a is greater or smaller than the absolute value of the difference between d and b, and the difference between these two absolute values is found to be significant ($p < .05$). Such nonparametric assessments without making any assumption on the distribution of vessels are more realistic, and "outliers" are shown in box-plot presentation of vessels before treatment.

CAM can be used for transport studies as the membrane can act as in situ transport barrier. However, for such studies, it is important to measure the physical characteristics of CAM such as thickness and surface area of CAM (Figure 1). Measurements of CAM diameters can be carried out by determining a few diameters using a pair of calipers. The surface characterization of CAM allowed extrapolations on the permeation of drug transported across CAM and ensured equivalence between the CAMs of control and experimental groups. Measurement of thickness can be carried out after sacrificing the egg. If thickness and area are statistically non-significant among the test groups, it can be concluded that drugs with different uptake rates are affected by differences owing to the hydrophilicity, hydrophobicity, or dosage form, depending on the design of the studies (Saw et al., 2005; 2006).

Despite the criticisms, CAM has been identified as a very useful alternative to animals testing for various biomedical studies such as in angiogenesis and antiangiogenesis research and in the development of pharmaceutical formulations during the past decade. Numerous time-dependent scores of irritants on CAM were validated to demonstrate its potential as alternative to the Draize test (Luepke, 1985). CAM has been used extensively for irritancy testing of chemicals/cosmetics. The full potential of CAM for use in formulation development can be realized with proper control of variables.

SUMMARY

The use of in vivo CAM for drug screening and drug effects, for evaluating formulations/dosage forms of different drugs, and for transport studies have been reported. Compared with conventional methods of using animal models or the Draize test, CAM provides an attractive alternative in vivo model for use in pharmaceutical formulation development, particularly at the preformulation stage. With appropriate control of experimental variables in CAM models, quantitative assessment of different formulations can be carried out at much lower cost with greater efficiency and faster measurements.

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