Review

DOI: 10.5582/ddt.2018.01079

Models for acne: A comprehensive study

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Summary **Acne vulgaris (AV) is the familiar chronic skin ailment affecting most of the individuals. This multifarious, disease involves the bacterium gram-positive, anaerobic *Propionibacterium acnes* (*P. acnes*) which resides on skin microflora, and participated in acne inflammation and acne lesions. The object of this review is to discuss presently available *in vitro*, *ex vivo*, and *in vivo* models to evaluate the cosmetic formulations that are developed for dealing and prevention of acne formation. These various available models offer new chances for further research on biologically active materials, drugs & pharmaceutical as well as cosmetics for acne treatment.**

Keywords: Acne, *Propionibacterium acnes*, pilosebaceous unit, sebaceous gland, animal models

1. Introduction

Acne, a multifarious chronic inflammatory state, is said to be happening within a pilosebaceous unit (PSU) including hair, hair follicles, sebaceous gland (SG) of the skin, characterized by non-inflammatory lesions-blackheads, whiteheads and inflammatory lesions-papules, pustul, nobless, nodules, and cysts (*1-3*). Acne is associated mostly with *P. acnes* which produces inflammation *via* release of extracellular enzymatic products like proteases, lipases, and hyaluronidases. About 94-95% of the pubertal population, 20-40% of adults and < 25% of women suffered from acne. Even after 25 years of age, women can suffer from is termed as adult female acne (*4-6*). Primarily four causes are critically responsible for the growth of acne lesion as shown in Figure 1 (*7*).

Some other factors are also responsible either alone or in combination for the formation of acne such as heredity, hormones, diet, and other bacterial species. This is well known that there are more chances for the children to have acne if their parents had acne in their young age, some heredity characters and genes which carry the similar information from parents to children. In another case, the various sex hormones like estrogens, androgens and tyrosine kinase hormones, corticotropin-releasing hormone, adrenocorticotropic hormone, glucocorticoid, and melanocortins are required to regulate SG activity. The androgen hormones produced during the adulthood activate the secretion of the oil glands and creates an obstacle in the pores resulting in the progression of blackhead and acne (*8-10*). In general, SG is major responsible part causing disease due to the existence of the enzyme necessary for the formation of various hormones, cytokines and unique lipid mixture of squalene, wax esters, triglycerides and sebum (by the holocrine process) (*11-13*). The structure of all SG found on different skin parts are similar but their nature and the regulation of secretions and secretory process are different (*14,15*). The larger part of SG in hair-covered areas is connected with hair follicles are known as pilosebaceous (PS) glands. The SG of the mucosal margin and modified skin are sovereign of hair follicles (*16*). Excess sebum secretion, abnormal differentiation and desquamation (the shedding) of follicular keratinocyte are some other responsible factors for acne (*17*). The upsurge of the microcomedo as a result of free fatty acid production is primary lesion which has been further developed as inflammatory along with non-inflammatory lesions (*18,19*). In other cases, the various species of bacteria *P. acnes*, *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* (*S. agalactiae*), *Klebsiella pneumoniae* (*K. pneumoniae*) *etc*. has also been for the hyperactivity of the SG and ultimately acne (*20*). *P. acnes* are the major causative bacteria which by stimulating androgen receptor to increase the androgen

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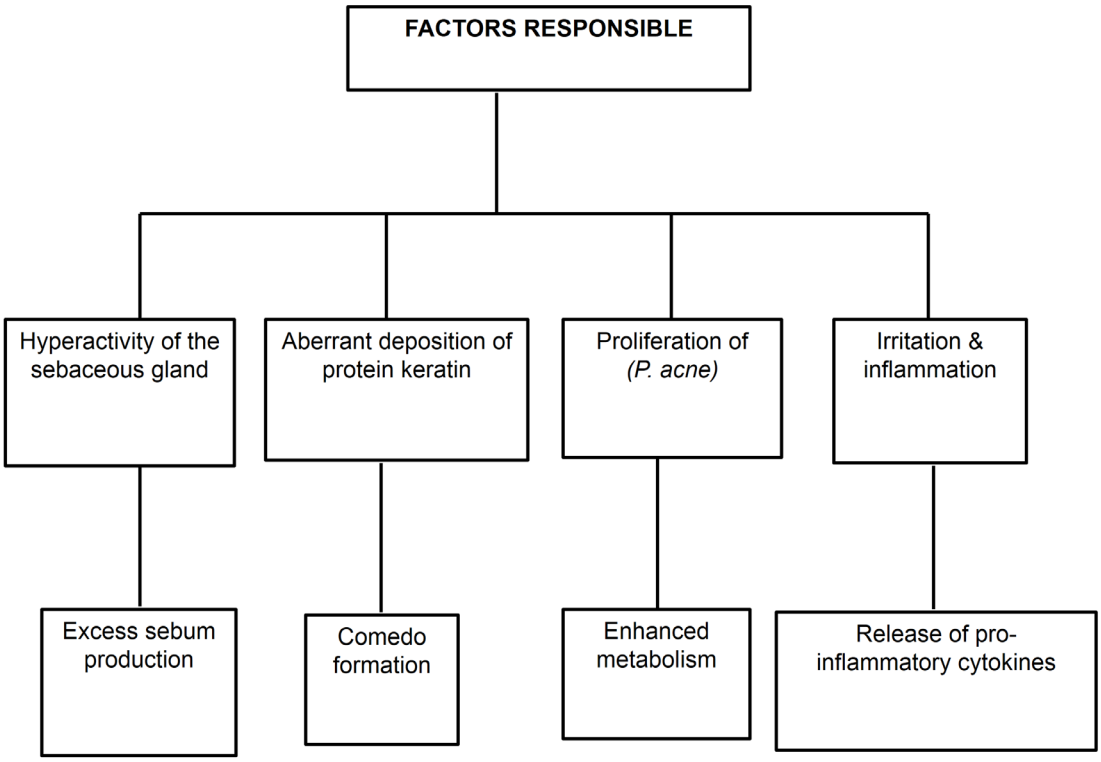

Figure 1. Factors responsible for acne.

secretion *via* androgenic hormone which induces the sebum production, keratinization, and colonization and finally induction of the inborn immune responses which cause inflammation (*21-26*). *P. acnes* also contribute to inflammation *via* activating various chemotactic factors and activate the inflammatory cascade through Toll-like receptors (*27*).

2. Mechanism of acne formation

The acne formation initiates with the overactive SG, follicular hyperkeratinization, immunological changes, and plugging of the infundibulum facilitating *P. acnes*, bacteria colonization, and inflammation (*28*). Increased sebum that is the major source of nutrients for *P. acnes* production is an important participating cause of the acne formation (*29-31*). The mechanism of acne formation should be understood for the design and estimation of the formulation for the effective treatment of acne. Hence, the detail pathogenic factors and process of acne formation is elucidated in Figure 2 and 3.

The various pathogenic factors blamed for the development of acne include the PSU, hyperkeratinization and undue sebum production, discharge inflammatory mediators, and inflammatory infiltrates (causes the progress of growing steps resulting in severity). *P. acnes* bacteria initiate the formation of propionic and acetic acid, thus resulting in the metabolism of sebaceous triglycerides into fatty acids. This results in the irritation of the follicular wall and surrounding dermis (*2,32*). *S. epidermidis*, an anaerobic microorganism, is another causative microorganism involved in acne pathogenesis and produces fatty acid modifying enzyme that forms cholesterol by the fatty acid esterification in the skin (*33,34*). *S. Aureus*, gram-positive rod-shaped bacteria, invade the skin and start producing extracellular enzymes such as lipases, proteases, hyaluronidases, and collagenase. These enzymes cause tissue injury and spread the pathogen into the deeper tissues (*35,36*). *S. agalactiae*, gram-positive coccus bacteria, is also involved in the development of *P. acnes*. The capsular polysaccharides and the pore-forming toxins of *S. agalactiae* are the key factors of *S. agalactiae* (*37*). *K. pneumoniae*, gram-negative bacteria present on the skin flora, causes infection in acne patients undergoing long-term antibiotic therapy. The malicious factors of *K. Pneumoniae* is capsular polysaccharides, siderophores, and adhesins, this virulence produces seditious acne lesions papules, pustules (*38*).

Various models for the antiacne analyze of the drugs and drug carrier are available, but not a single article available which is fully devoted to models for antiacne activities (*39*). Therefore, with the present review article, the authors try to gather and compile the information regarding *in vitro*, *ex vivo*, and *in vivo* models applicable to study acne and are reviewed in the subsequent section (Figure 4).

3. *In vitro* models for acne

In vitro acne models used for the study are organ culture, monolayer culture, follicle model and testosterone-

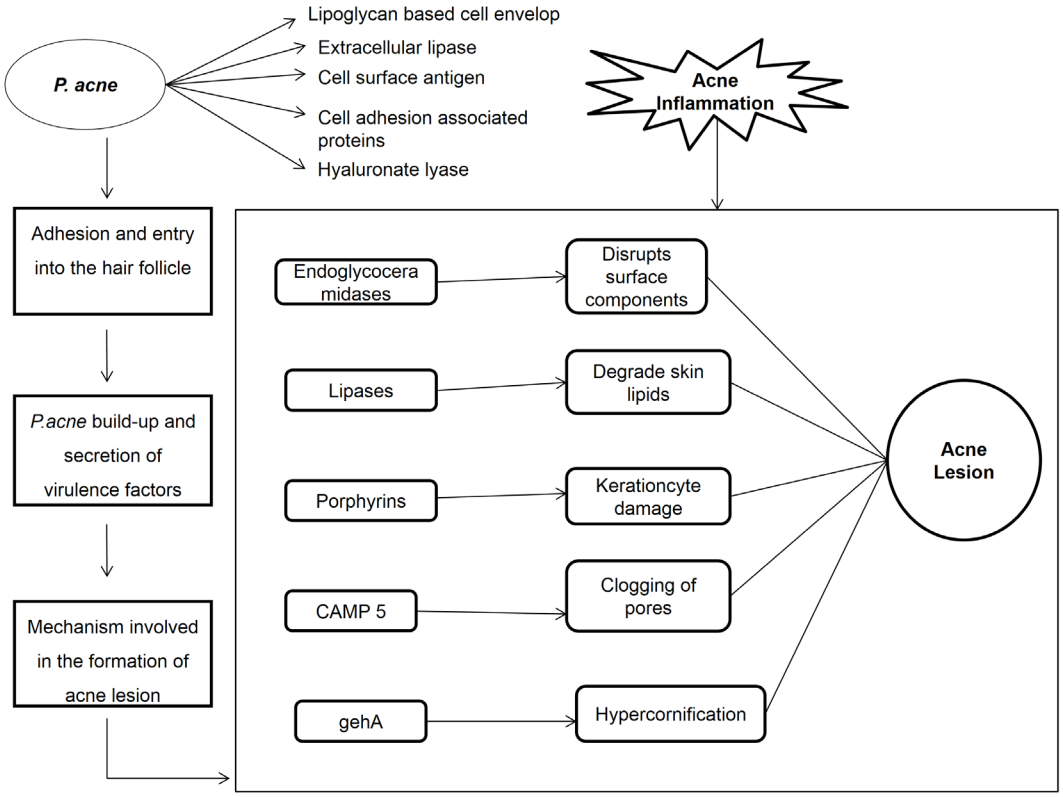

Figure 2. Mechanism of acne formation.

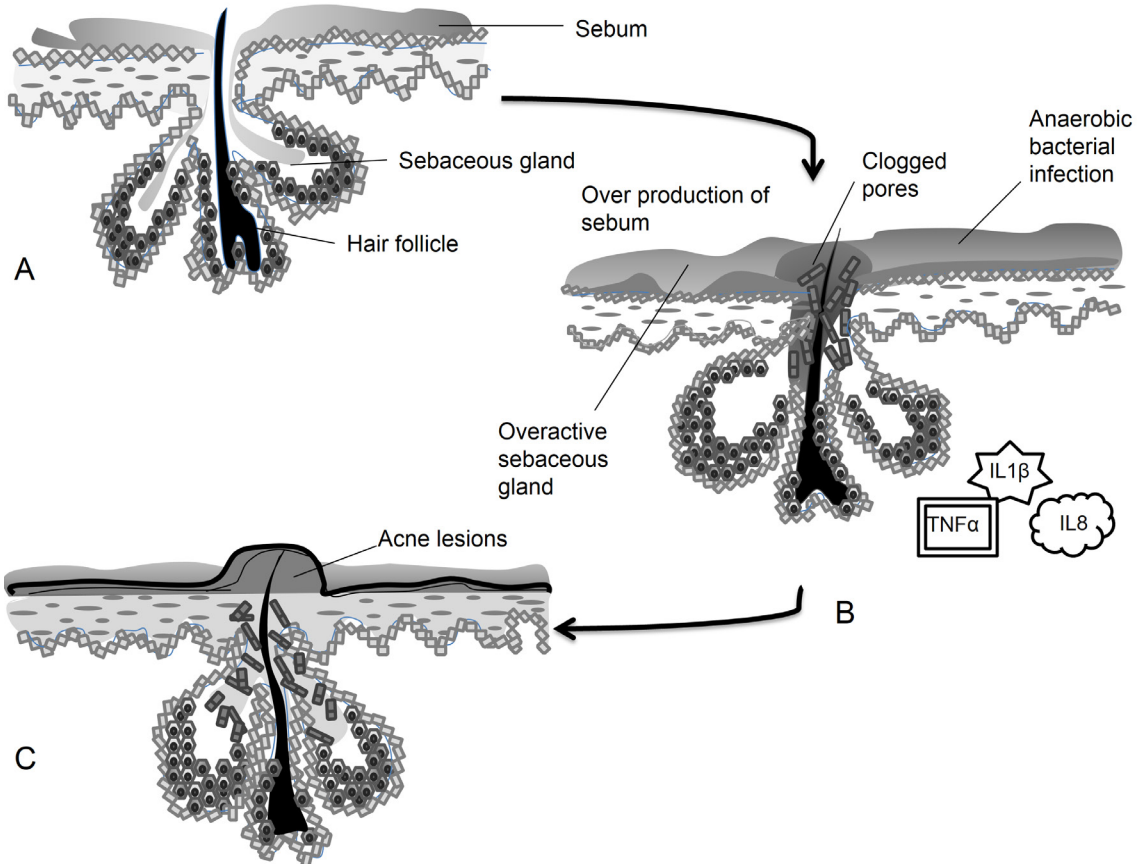

Figure 3. Causative pathogenic factors in acne: (1) Pilosebaceous unit. (2) Mechanism and effects of clogging pore. (3) Inflammatory infiltrates in acne formation.

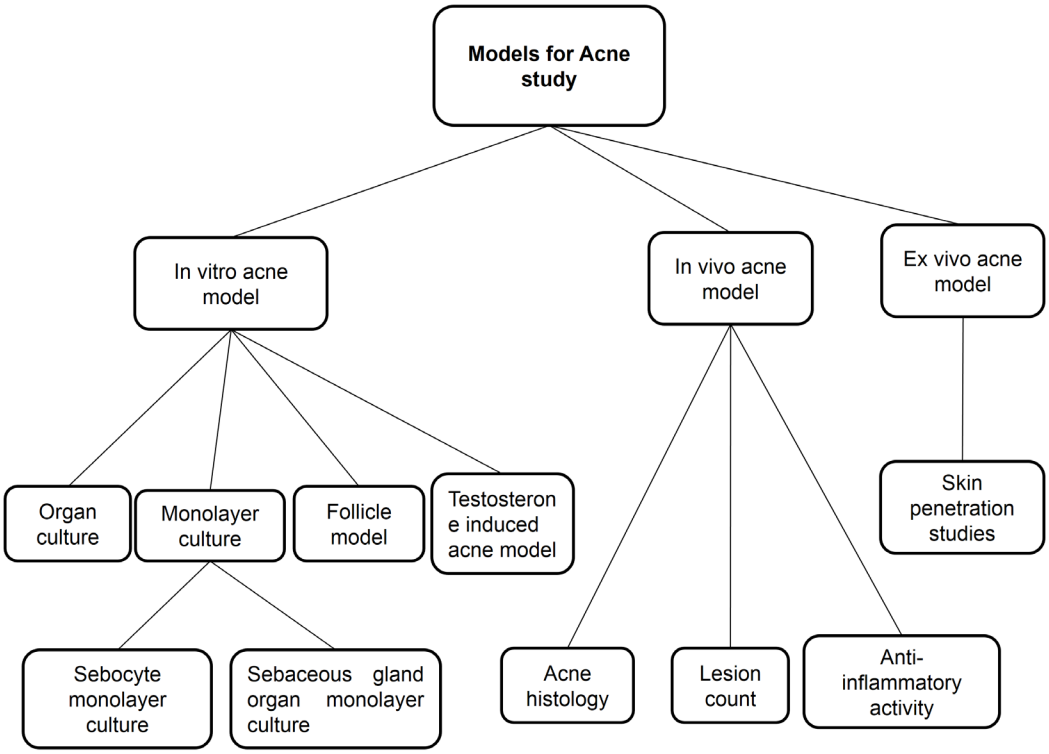

Figure 4. Models for acne study.

induced model. Though, no *in vitro* model is presented which can be used to study the complete perplexity of lesion formation. In *in vitro* organ culture model, the growth and preservation of any tissue, organ, and parts of organ allow the demarcation of that tissue and organ (*40*). In monolayer culture, cells are grown in a single layer on a culture medium in a Petri dish and prepared by the enzymatic separation of organ fragments. Cells in the monolayer culture need a substrate, which should be biologically inert, nontoxic and both the substrate and cells should be compatible with each other, otherwise, the culture will not endure and causes anomalous morphological changes (*41*). While in sebocytes (specialized epithelial cells) monolayer culture the sebocytes are used. These cells release their cellular content by degradation and by rupturing the cell membrane. The lipid synthesis in sebocytes is characterized by the rapid cell multiplication and differentiation of sebocytes. Cultured sebocytes are producing fewer amounts of wax esters and Squalene than the freshly isolated and *in vivo* SG (*42*).

3.1. *SG organ culture*

These models are important to explore the pathophysiology of human skin disorders which involves the SG, *i.e.* sebostasis, seborrhea, and acne. These models are a valuable means to develop and evaluate cosmetics and drug products. Human SG organ culture models highlight the role of SG and explain their functions in skin homeostasis. This model offers numerous benefits in comparison to sebocytes monolayer culture (*40*). These models are developed in isolation of SG by dissection of human skin (surgical waste, donor, cadaver, *etc*.) or by micro-dissection and shearing. The isolated specimens are transferred quickly into Dulbecco's modified Eagle's medium. The epidermal layers of the isolated specimen are separated and remaining dermis parts are directly placed in the growth medium. Intact SGs with/without dermal/epidermal components are utilized to measure lipogenesis rate in subjects with acne and the effect of substrates on lipid rates. To cultivate SG-derived cells, the isolated SGs were seeded in Petri dishes on a 3T3-cell feeder layer and complete culture medium. The cultures were incubated. The proliferation and lipid formation can be simultaneously examined in the basal and differentiated cell layer (*43*). An additional advantage lies over animal model in the fact that delivery of potential sebum suppression compound to the gland is not hindered, so absolute biological efficacy can be evaluated. The advantages of SG organ culture are distinct, although they involve the procedure which is time-consuming.

The SG *in situ* model is applicable for the antiacne studies using the culture of SGs isolated from animals. Toyoda and Morohashi performed organ culture on SG obtained from mice to compute the effects of neuropeptides (calcitonin gene-related peptide, substance P, vasoactive intestinal polypeptide, and neuropeptide Y) along with nerve growth factor. SG treated with substance P resulted in the accelerated lipid synthesis over the control glands by escalating the rate of

Table 1. Reported SG experimental models

Experimental model	*Ref.*
Isolation of SGs after enzymatic dissociation for the cultivation of human sebocytes (HSs).	*45*
Introduced the condition to maintain SG ex vivo.	*46*
A modified version of the technique reported by Karasek, 1986. In this experiment the second 0.4-mm dermal section is used as a source of HSs after removal of epidermis and dermis layer (about 0.4 mm thick) of facial skin.	*47*
In vitro cultivation of human SG-derived cells. HS monolayer cultures as growth from the periphery of SG organ cultures were obtained.	*48*
Xia *et al*. 2009 developed the 1° sebocyte cultures by omitting the 3 T3 fibroblast layer, and 2° culture medium supplemented with delipidized serum and serum-free keratinocyte basal medium.	*49*
Zouboulis *et al*. 2009 described the method for generation of an immortalized sebocyte cell line "SZ95" by transfecting human facial sebocytes with Simian virus-40 large T antigen. This cell showed similar morphologic, phenotypic and functional characteristics of normal HSs. Thiboutot *et al.* Immortalized human SG cell line (SEB-1) by applying the transfection system and Zouboulis *et al*. immortalized SG cell line (Seb-E6E7) by the introduction of HPV16 E6 and E7 genes.	*50-52*

differentiation and proliferation postulating that stress which leads to acne (*44*). SG experimental models are reported in Table 1.

3.2. *Sebocyte monolayer culture*

Although the advantages of SG organ culture are evident, the procedure is time-consuming and depends on continued sources of viable tissue for additional glands. Cell culture offers an alternative over organ culture maintenance, a massive amount of cells can be processed and frozen, allowing multiple experiments to be performed on a similar cell lineage. Several laboratories have productively cultured HS cultures, in the presence of fibroblast support and in serum-free medium. This allows observing the effects of serum and growth factors. HS culture is a well-formed model to change local environmental growth conditions, evaluate lipogenesis inhibitors in a higher throughput mode than that which would normally be obtained through the utilization of organ culture and facilitate the study of cell metabolism, specifically to the androgen metabolism and lipid synthesis. In sebocytes, the synthesis of lipid is dose-dependent (*7*). Sebocytes monolayer culture offers an advantage that maximum cells can be processed and frozen which is used for performing multiple experiments on a similar cell lineage. There are two procedures for generating sebocytes monolayer cultures: (a) first one is explant outgrowth method where isolated SG are attached to culture plates followed by addition of growth medium, following incubation for several days, visible outgrowths (proliferative basal sebocytes) are seen from the glands; (b) second one is SG digestion method which involves digestion of SGs by limited trypsin proteolysis and released cells are plated in mass culture upon a fibroblast feeder layer. After a few days, there is a replication of attached cells into small colonies that can be further used as cloned cells.

3.2.1. *Rat preputial sebocyte monolayer culture*

The preputial gland of rodent possibly is employed as a model for the human SG. These specialized glands open to the surface with a surface of the urethral meatus, and secretions are included in territorial marking and mating behavior. Rat preputial glands are utilized to produce monolayer cultures that can be isolated, digested, and cultured upon a fibroblast nutritive layer (*53,54*). The preputial monolayer cultures of these cells are a suitable model for evaluating the hormone effects on sebaceous development and growth. The effects of estrogen on preputial cell behavior and determination of the cultured cells along with the action of androgen and effect of peroxisome proliferator-activated receptor (PPAR) has been reported (*55,56*). Preputial cells differentiate in a similar process, but to an overall lesser extent than human sebocytes (*57*). Preputial cells cultured *in vitro* lack the existence of PPAR ligands that will induce the formation of the oily or lipid droplet (*58*).

3.3. *Follicle model*

The follicle model is used to appraise the context among sebocytes, keratinocytes and *P. acnes*. *P. acnes* colonizes on superficial skin and PSU. For determining the connection between sebocytes, keratinocytes and *P. acnes*, preserved sebocytes (SZ95) and keratinocytes (HPV-KER) were cultured in the same chamber which is divided by a permeable polyester membrane. The permeable polyester membrane avoids the dispersion of soluble molecules among the chambers. In the healthy PSU, *P. acnes* can only be found in the hair follicular cells (*59*).

3.4. *Squalene oxidation model*

Squalene oxidation models have been developed as the skin tissue engineering models for the *in vitro* testing of acne. These models are utilized for determining the sebum composition as well as the role of oxidative derivatives of squalene (*60*). These models are developed

by the squalene oxidation under controlled atmosphere *i.e*., by oxygen, ultraviolet irradiation and controlled incubation at prescribed conditions. The oxidized squalene derivatives have been analyzed and measured by GCMS and NMR at the predecided condition. The reconstructed human epidermis (RHE) models are also developed which are depended on the use of oxidative derivatives of squalene. On this morphology of the RHE were studied and specific markers such as inflammatory cytokines were measured. This model is used to calculate the drug *in vitro* activity and their formulations to inhibit acne development (*61*).

3.5. *Testosterone-induced acne model (TIAM)*

Testosterone is, a steroidal hormone, accountable for different forms of acne. The conversion of testosterone to dihydrotestosterone, stimulate keratin through its follicular receptor (*62*). Both the dihydrotestosterone and testosterone are the causes of acne because of the increased activity and size of SG. The TIAM is prominent for determining the efficacy and biocompatibility of the various components (*4*) (Figure 5).

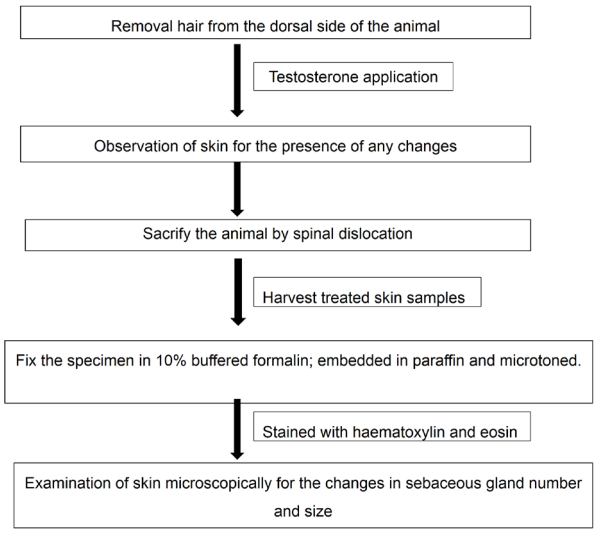

Figure 5. In-vitro testosterone acne induced model.

4. *In vivo* models for acne

These models have been utilized to predict the efficacy of the drug in the treatment of that particular disease (*63*). Recently, animal models have been used for drug development in an exponential manner. The *in-vivo* model has the same physiology as the events of human anagenesis and advantageous to appraise the anti-acnegenic nature of the formulation. These models imitate both human skin diseases and conditions. There are different human and animal models are available for acnegenesis, such as the Mexican hairless dog, rabbit ear assay and Rhino mouse model that closely resembles comedogenesis (*64*).

Skin irritation is the culmination of a complex inflammatory process involving epidermal keratinocytes, dermal fibroblasts, endothelial cells, and annexing leukocytes in which early symptoms may consist of swelling, redness, and itching continuing to scaling and erythema (*7*). Histologically, irritation involves both the epidermis and dermis with inflammatory infiltrates entering from the microvasculature and chemical mediators being released from the different types of cells. The important task of the keratinocytes is not only to provide a barrier and integral structure of the PS follicle but also to participate as the essential task in the inflammatory process through the release/response to surrounding cytokines. Alternatively, the incite inflammation is the key role of bacteria in acne. *In vivo* acne models allow studying and determining the lesion count, the effect of the drug on bacteria, comedogenicity, histology study, and other anti-acne activity.

Two types of inflammatory animal models used for ascertaining the immunostimulatory, pathogenic and pro-inflammatory properties. These models included; 1) an acute inflammation model, induced by drug topically and 2) a chronic skin inflammation model partially relevant to AV, which is developed with the bacterial intradermal injection into the mouse's ear. The detailed procedure for the estimation of anti-acne inflammatory activity and ear thickness as well as acne histology and lesion count shown in Figure 6 and 7, respectively (*65-68*).

Cytotoxins are compounds that stimulate chemotactic activity and are produced by *P. acnes* and other microorganisms in the follicle, which attract neutrophils (*69*). These chemical mediators can then go on to activate macrophages, neutrophils, natural killer cells and other cells inducing the production of additional cytokines. Major inflammation mediators include; histamine, leukotrienes, interleukins, prostaglandins, and neuropeptides. Neutrophils then produce enzymes and generate free oxygen radicals that will result in the disruption of the follicle. Inflamed parts such as pustules, papules, and nodules are developed when comedones rupture and their contents extruded into the dermis rather than above the skin surface. For analysis purposes, the live *P. acnes* (ATCC 6919) will be injected into the mouse's ear. The ear is then cut out on day 1 to day 5 after bacterial injection, and homogenized with the tissue grinder, in 0.9% saline solution. Then supernatant centrifuged for 10 min at 2000 × g to separate out the clear liquid and then cytokine is measured. The TNF-α and IL-1β concentrations are determined with the help of ELISA kits (*70,71*).

5. Skin penetration studies

Ex vivo conditions permit examination on an organism's cells or tissues under more controlled conditions than is attainable *in vivo* testing, to the detriment of modifying the "natural" environment. These studies help to observe

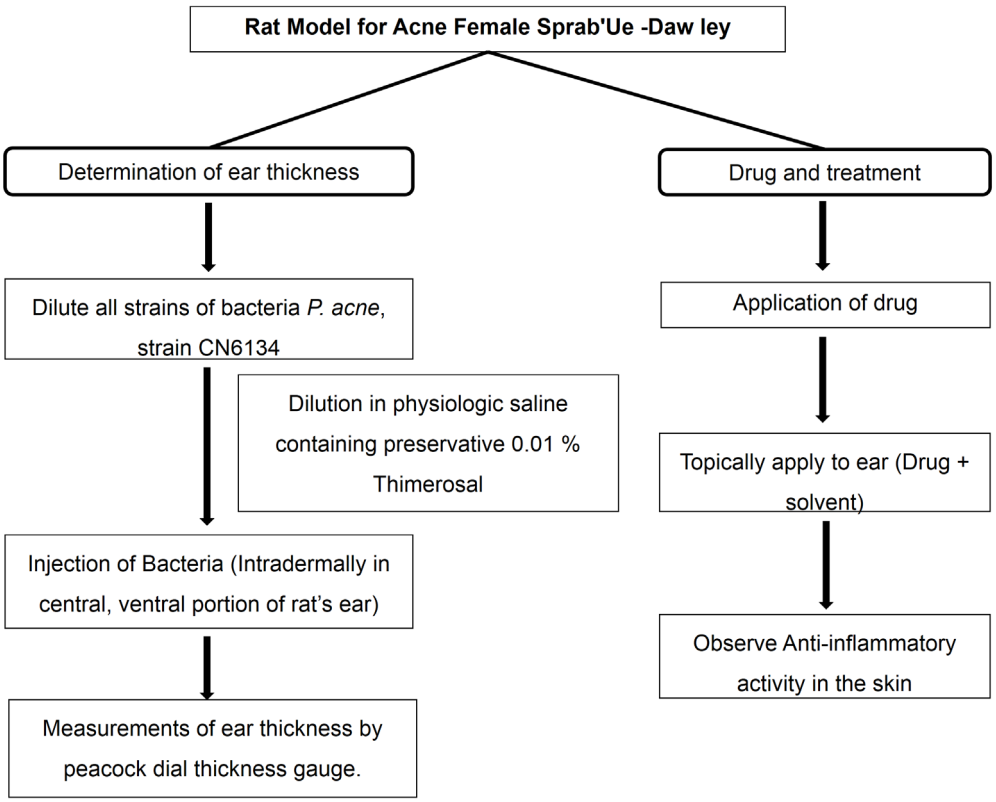

Figure 6. Rat model for acne for determination of anti-inflammatory activity and ear thickness.

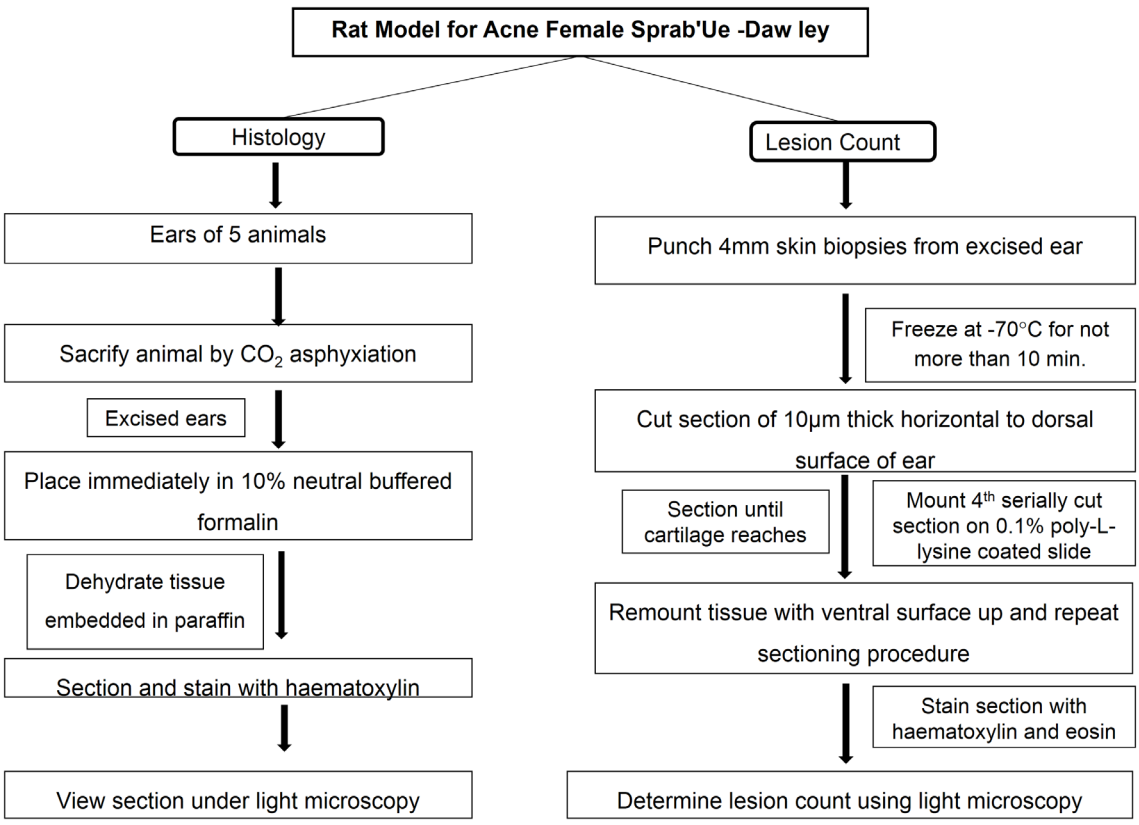

Figure 7. Rat Model for acne histology and lesion count.

the permeation rate of specific compounds through the animal skin (*72*). *Ex vivo* models are economical, effortlessly obtained, but not suitable to determine the long-term experiments or the heat sink effect. The studies can be summarized with the help of Figure 8. For *in vitro* permeation study, the permeation rate of the carrier through the skin, along with drug entrapped will determine by using egg membrane (Figure 9) (*73*).

6. Estimation of antimicrobial (ATM) activity

The activity of ATM component is measured by

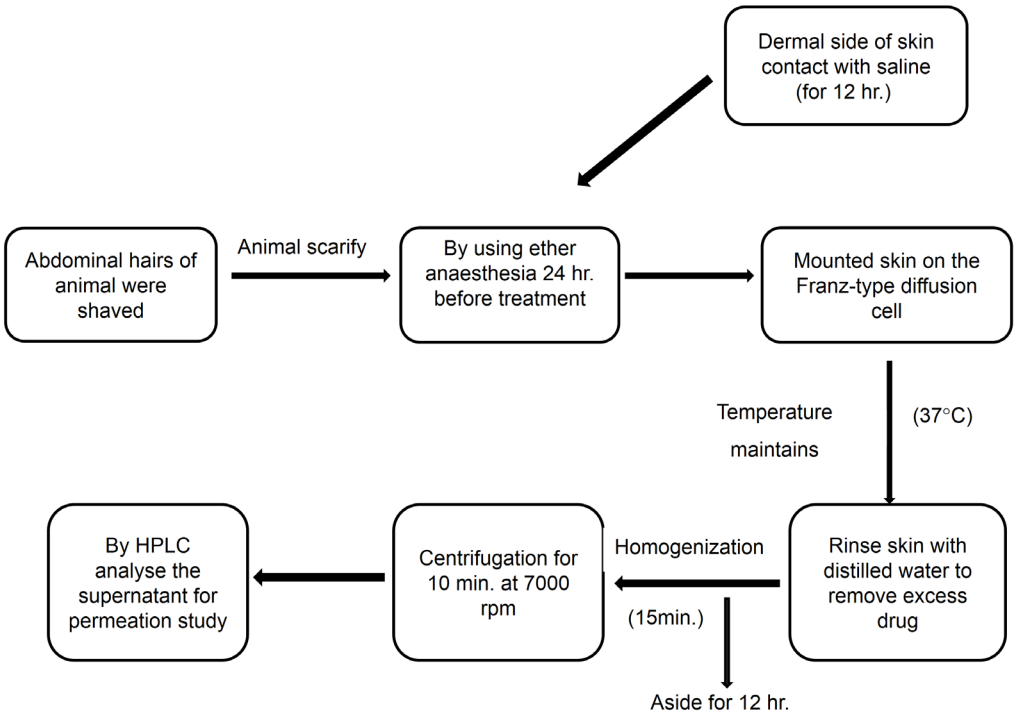

Figure 8. *Ex-vivo* skin penetration studies.

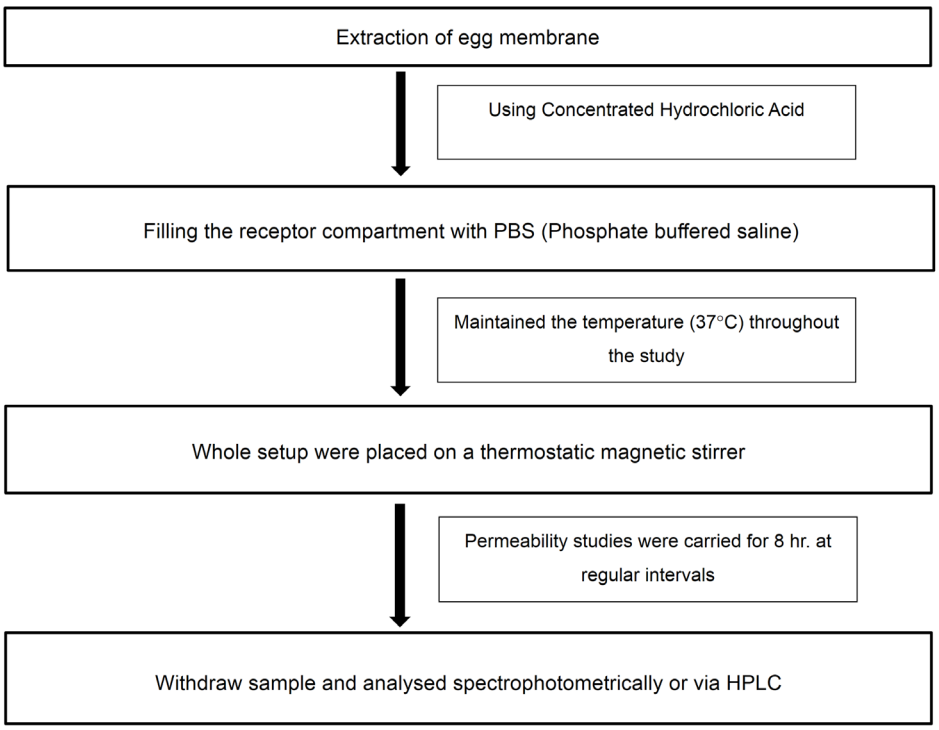

Figure 9. *In-vitro* model for skin permeation study using egg membrane.

determining the minimum inhibitory concentration (MIC). The MIC is the smallest concentration of an ATM agent that will reduce the growth and development of a definite type of organism after sub-culture on antibiotic-free medium. MIC is employed to confirm organism resistance and *in vitro* potency of the new ATMs and it is regarded as the 'gold standard' for the determination of susceptibility of the organism to ATMs. The methods used for determining the MIC of the therapeutic agent are discussed below (*4,74*).

6.1. *ATM assay*

For ATM assay, two microbial varieties *S. aureus* (MTCC 96) and *P. acnes* (MTCC 1951) is usually used. The activities of compound and formulations have been evaluated against *P. acnes* in agar and *S. aureus* in pre-warmed agar plates (APLs). For this microbial cultures should preserve in their suitable agar slants at 4°C. This is used as stock cultures (*75*). About 60 µL of each component is placed on sterile paper discs (8mm diameter) kept above the APL surface. Plates were cultured at 37°C for 48 h (*72*). The ATM potential of the test compound is determined by the measurement of the zone of inhibition (ZOI) diameter (*2,76*).

6.2. *Tube dilution technique (test tube method)*

The tube dilution method is referred to as the standard method for the assessment of microbial resistance against a specific ATM agent. In test tube method, antibiotic dilutions are prepared in a liquid medium. In the series of culture tube (filled with microbial growth medium) (*67*) the test compound (variation in concentrations of test compound) is added. Incubate the culture tube. If there is no turbidity found it represents inhibition in the tube (*7,77*).

6.3. *Agar diffusion method*

It is applied for the assessment of the ATM MIC. Both the nutrient agar and nutrient broth medium have been used for the bacterial culture and for the assessment of ATM potency (*78*). To the Petri plates containing an agar medium, ATM agent is added to filter paper disc and places it in contact with the agar surface (*46*). During the incubation period, the ZOI will be formed, after the diffusion of the test compound onto the APL from the filter paper. The effectiveness of the therapeutic agent is determined by the diameter of ZOI (*5,47,75*).

6.4. *Anti-androgen hypersecretion of sebum*

The fuzzy rat model is selected to study the assessment of hyperplastic SG and sebum hypersecretion. This model is a genetic mutant variety between hairy albino and hairless rats. It is used to determine the effects of molecules or drugs on the reduction of glandular and ductal hyperplasia in human acne. For this, a sample has been diminished into the appropriate vehicles (*i.e*. water, alcohol) *etc*. and applied on the back of all male rats for 2 months, except the control and the vehicle receiving groups of rats. The size of the sebaceous glandular lobes (GLs) and ducts will be quantified in both the fragmented epidermal preparations and frozen sections of skin. After 8 weeks it is evaluated by staining with osmium-potassium dichromate solution. The calculation can be done by the number of bromodeoxyuridine (BdU) positive cells in the GLs in split-skin tissues stained with BdU immunochemistry (*48*).

6.5. *Ex vivo pig skin colonization*

The segment of hair free pigskin has been sterilized in 70% alcohol were clipped and infect with bacterial or fungal inoculum. Then the skin pieces (10 per group) are treated with 100 µL of ATM agent. The treated skin has been incubated for 24h at 35 ± 2°C under humidified conditions. Colonies were counted by swabbing the skin surface with a sterile cotton-tipped applicator soaked in 1mL of sterile saline supplemented with ATM inhibitor. Then after the sampling solutions is diluted and poured into the plates for colony counts. If there were no viable colonies observed, this is assigned as low detection value (*49,79*).

7. Conclusion

Recently, a remarkable headway has been attained in delineating the models available for the acne. In conclusion, the culture of the human SG cell model offers unique possibilities for further fascinating investigations into the role and importance of the SG in the acne development. Moreover, these models which are an adequate tool for evaluation of the pharmacological properties of new anti-acne compounds. The sebum model sustains *P. acnes* progresses and biofilm formation and mimics some important phases of the PSU. Multiple models are available for the identification of technologies for effective acne treatment. By using these models, it is possible to estimate the mechanism of action of lead compounds. More complex culture systems, including three-dimensional models, are under development. The above-discussed approaches are very useful in further advancement in the selection of efficacious drug to the development of drug delivery systems for the acne treatment.

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(Received December 10, 2018; Revised December 18, 2018; Accepted December 29, 2018)