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REVIEW

In Vitro models for evaluating anti-aging formulations: Biomolecular efficacy, quality, and safety assessment

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ABSTRACT

Introduction: The advancement of anti-aging formulations, both topical and injectable, requires robust preclinical strategies to ensure their biological effectiveness, safety, and quality. In vitro models have become pivotal in this process, offering ethically sound and mechanistically insightful platforms that reduce reliance on animal testing.
Objectives: This review critically examines the spectrum of in vitro systems currently employed for the preclinical evaluation of anti-aging formulations.
Methods: We analyzed the range of in vitro systems currently used, from conventional 2D cultures to sophisticated 3D skin equivalents and organ-on-chip technologies, to evaluate their contribution to preclinical testing.
Results: These models contribute to understanding biomo lecular pathways, formulation stability, and toxicological profiles.
Conclusions: Regulatory trends and translational challenges are discussed, highlighting the integration of in vitro methods with omics and computational tools as a promising frontier in aesthetic medicine.

Key words: Anti-aging formulations, *in vitro* models, 2D skin models, 3D skin models, organ-on-chip

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Introduction

The growing societal emphasis on longevity and aesthetic wellness has intensified the demand for scientifically validated anti-aging interventions, particularly those targeting the skin. As a dynamic and multifunctional organ, the skin not only reflects chronological aging but also serves as a primary site for therapeutic and cosmetic applications. Innovations in formulation science, ranging from bioactive topicals to injectable biostimulants, must be supported by rigorous preclinical evaluation to substantiate claims and ensure safety[1,2]. Historically, animal models have played a central role in assessing biological activity and toxicity. However, ethical imperatives, regulatory shifts, and translational limitations have catalyzed a paradigm shift toward alternative testing strategies[1-3]. *In vitro* models now occupy a central position in early-stage product development, enabling precise interrogation of cellular responses, mechanistic pathways, and formulation behavior under controlled conditions[1,4-6]. Guided by the principles of replacement, reduction, and refinement (3Rs)[7,8], these models offer a scientifically robust and ethically responsible framework for innovation in anti-aging medicine[1,2,6].

In this evolving landscape, it is essential to distinguish between cosmetic formulations, typically regulated under frameworks such as EU Regulation 1223/2009, and medical or injectable products, which may fall under medical device or pharmaceutical legislation (e.g., MDR 2017/745 or 21 CFR Part 820). While both categories benefit from *in vitro* testing, the regulatory expectations, safety endpoints, and translational goals differ significantly. Therefore, selecting appropriate *in vitro* models requires careful alignment with the intended application, whether cosmetic (e.g., wrinkle reduction, hydration) or medical (e.g., dermal fillers, regenerative therapies).

Search strategy and articles' selection

We conducted a non-systematic review using PubMed, Scopus, and Web of Science databases. We included peer-reviewed articles published between 2012 and 2025, focusing on *in vitro* models applied to anti-aging formulations. Inclusion criteria prioritized studies addressing biological efficacy, safety, and regulatory aspects. Non-English articles, duplicates, and publications lacking experimental relevance were excluded.

In vitro models in anti-aging research

The development of anti-aging products, whether topical or injectable, requires a nuanced understanding of their interactions with human skin at the cellular and molecular levels. *In vitro* models offer a versatile and ethically sound approach to simulate skin physiology, enabling researchers to investigate mechanisms of action, compound penetration, and safety profiles prior to clinical testing[9]. These models vary in complexity, ranging from basic monolayer cultures to engineered three-dimensional (3D) skin constructs and dynamic microphysiological platforms[10,11]. Each system serves distinct experimental purposes and presents specific advantages depending on the formulation type and research objectives[2,12-14] (Figure 1).

To facilitate comparison and model selection, Table 1 summarizes the key features of each *in vitro* platform, including complexity, advantages, limitations, and typical applications in anti-aging research.

2D Cell cultures

2D cell culture systems remain foundational in dermatological research due to their simplicity, reproducibility, and suitability for high-throughput screening[2,15-18]. Typically involving human dermal fibroblasts or keratinocytes grown on flat substrates, these models allow for rapid assessment of cellular responses to bioactive compounds[1-5,19-21]. Fibroblasts are particularly relevant in anti-aging studies, given their role in synthesizing extracellular matrix (ECM) components such as collagen and elastin[6,7]. Experimental endpoints often include cell proliferation, oxidative stress markers, and modulation of senescence-associated pathways[8,9]. Keratinocytes, on the other hand, are used to evaluate epidermal renewal, barrier integrity, and inflammatory responses, especially under conditions mimicking environmental stressors like UV exposure or pollution[10-12].

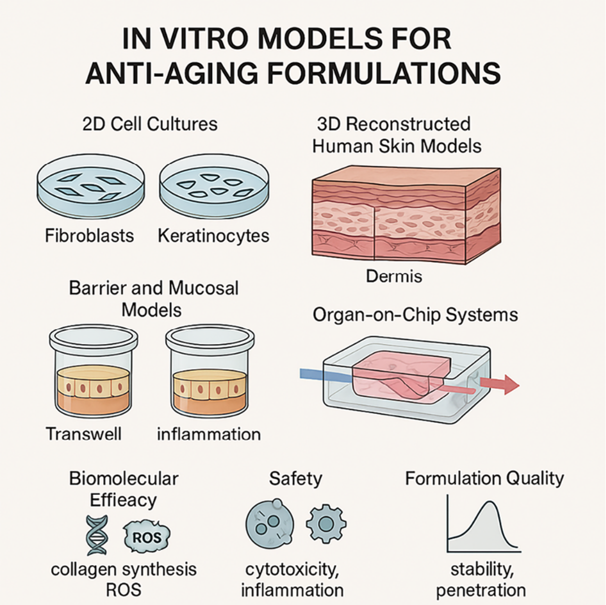

Figure 1. Schematic overview of *in vitro* models used for the evaluation of anti-aging formulations. The diagram illustrates four main categories: 2D cell cultures for high-throughput screening and mechanistic studies; 3D reconstructed human skin models for assessing barrier function and long-term efficacy; barrier and mucosal models for injectable formulations and tissue compatibility; and organ-on-chip systems for dynamic, physiologically relevant testing. These models support the assessment of biomolecular efficacy, safety and toxicology, and formulation quality.

Despite their utility, 2D cultures lack the architectural complexity of native skin, limiting their predictive value for *in vivo* outcomes[5,13,14]. Prolonged culture may also induce phenotypic drift, underscoring the need for complementary models in later stages of product development.

3D Reconstructed human skin models

3D reconstructed skin models represent a significant advancement of *in vitro* testing, offering a more physiologically relevant alternative to traditional 2D systems[2,15]. These constructs typically consist of primary human keratinocytes layered over a fibroblast-populated dermal matrix, cultured at the air–liquid interface to promote epidermal stratification and barrier formation[16,17].

In anti-aging research, 3D models enable the evaluation of long-term effects of topical treatments, including changes in epidermal thickness, collagen deposition, and expression of biomarkers linked to aging and inflammation[18,19]. They also facilitate studies on percutaneous absorption and irritation potential, aligning with regulatory guidelines for safety assessment[20,21,22].

Table 1. Comparative overview of in vitro models for anti-aging formulation testing.

Model Type	Complexity	Advantages	Limitations	Typical Applications
2D Cell Cultures	Low	Simple, cost-effective, high-throughput, mechanistic insights	Lack of tissue architecture, limited predictive value	Screening of actives, oxidative stress, senescence, proliferation assays
3D Reconstructed Skin Models	Medium	Mimic epidermal structure, barrier function, long-term exposure studies	No vasculature or immune cells, higher cost	Topical efficacy, irritation, collagen synthesis, biomarker expression
Barrier/Mucosal Models	Medium	Simulate epithelial interfaces, assess permeability and compatibility	Limited structural complexity, often lack full skin layers	Injectable formulation testing, mucosal irritation, cytokine release, tissue remodeling
Organ-on-Chip Systems	High	Dynamic microenvironment, multi-cellular, real-time monitoring	Technical complexity, limited standardization, high cost	Chronic exposure, systemic interaction, advanced efficacy and safety profiling

Advanced versions of these models incorporate senescent cells or are exposed to pro-aging stimuli (e.g., UV radiation, oxidative agents) to simulate aged skin conditions[1,3,23,24]. Their ability to support co-culture systems and time-course experiments makes them particularly valuable for testing complex formulations, including those delivered via nanocarriers or emulsions.

While 3D models offer enhanced biological relevance, they are more resource-intensive and may lack certain skin features such as vasculature or immune components. Nonetheless, they remain indispensable tools for bridging the gap between simplistic *in vitro* assays and clinical studies.

Barrier and mucosal models

While topical formulations primarily interact with the epidermal barrier, injectable anti-aging treatments, such as dermal fillers, mesotherapy agents, and biostimulatory compounds, are administered directly into or beneath the skin[25]. This route introduces unique challenges related to tissue compatibility, diffusion dynamics, and localized inflammatory responses[26,27].

To address these aspects, specialized *in vitro* models have been developed to replicate epithelial and mucosal barriers[28]. These systems often consist of monolayers or multilayers of epithelial or endothelial cells cultured on permeable membranes, such as Transwell® inserts. They allow for bidirectional analysis of compound transport and cellular responses, offering insights into how formulations behave once introduced into tissue-like environments[29,30].

Mucosal models are particularly relevant for formulations targeting perioral, periorbital, or intranasal regions, where the tissue is thinner and more permeable. These models may incorporate immune cells or fibroblasts to better simulate the native microenvironment, enabling the evaluation of irritation potential, cytokine release, and tissue remodeling effects[22].

In the context of injectable products, barrier models are used to assess biocompatibility, including cytotoxicity, apoptosis, and inflammatory signaling[23,24]. For example, hyaluronic acid-based fillers can be tested for their ability to maintain cell viability and avoid triggering immune activation[25]. Additionally, these models support studies on degradation kinetics and tissue integration, critical parameters for determining the longevity and safety of injectable treatments[26]. More advanced systems may include extracellular matrix components or hydrogel scaffolds to mimic the mechanical and biochemical properties of dermal tissue[27,30]. These enhancements improve the predictive accuracy of *in vitro* testing, particularly for formulations with complex delivery mechanisms or sustained-release profiles.

Although less commonly employed than full-thickness skin equivalents, barrier and mucosal models are gaining relevance in parallel with the rise of minimally invasive aesthetic procedures. As regulatory frameworks evolve, these models are expected to play an increasingly central role in preclinical evaluation of injectable anti-aging therapies[20,28].

Organ-on-Chip and microphysiological systems

Organ-on-chip (OoC) and microphysiological systems represent the cutting edge of *in vitro* modeling, offering dynamic platforms that closely replicate human tissue architecture and function. These technologies integrate microfluidics, biomaterials, and living cells to simulate physiological conditions such as perfusion, mechanical stress, and biochemical gradients[29,30].

In dermatological and anti-aging research, skin-on-chip models emerge as powerful tools for studying complex interactions between skin cells, extracellular matrix components, and therapeutic agents[22,31]. Unlike static cultures, OoC systems allow for continuous nutrient flow and waste removal, supporting long-term culture and more accurate modeling of chronic exposure scenarios. These platforms can incorporate multiple cell types, keratinocytes, fibroblasts, endothelial cells, and immune cells, in spatially organized compartments[23]. This enables the study of intercellular communication, immune responses, and age-related changes in skin biology[22-24]. Real-time monitoring of physiological parameters, such as transepithelial electrical resistance (TEER), oxygen consumption, and cytokine release, further enhances their analytical capabilities[29].

Skin-on-chip devices are particularly valuable for evaluating topical antioxidants, peptides, and retinoids under conditions that mimic real-life application, including mechanical stress and environmental exposure. Moreover, multi-organ configurations, such as coupling skin-on-chip with liver-on-chip, allow researchers to investigate systemic interactions and metabolic transformations of active compounds[25,26].

Despite their promise, OoC technologies face challenges related to device fabrication, standardization, and accessibility. However, ongoing advances in bioengineering and regulatory interest are paving the way for broader adoption. These systems offer a compelling alternative to animal models, combining biological relevance with analytical precision to support the next generation of personalized anti-aging therapies.

Key evaluation parameters

The preclinical evaluation of anti-aging formulations requires a multidimensional framework that integrates biological efficacy, safety, and formulation quality. Each of these domains encompasses specific endpoints and analytical strategies that, collectively, provide a comprehensive understanding of a product's performance[2,27]. The choice of evaluation parameters depends on several factors, including the type of formulation (topical or injectable), its intended mechanism of action, and the complexity of the employed *in vitro* model[28,29]. For example, a serum designed to stimulate collagen synthesis may be assessed using fibroblast-based assays, while a filler intended for subdermal injection may require barrier models to evaluate tissue compatibility and inflammatory potential.

This section outlines the three principal categories of evaluation:

- Biomolecular efficacy, which investigates how a formulation can modulate cellular pathways related to aging and regeneration.
- Safety and toxicology, which identifies potential adverse effects such as cytotoxicity, genotoxicity, and inflammation.
- Formulation quality, which examines physicochemical stability, release kinetics, and compatibility with biological tissues.

These parameters not only guide formulation refinement but also support regulatory submissions and claims substantiation. When integrated into modern *in vitro* workflows, they enable a rigorous and ethically responsible approach to product development in aesthetic and regenerative dermatology[30].

Biomolecular efficacy

Biomolecular efficacy refers to the formulation's capacity to modulate cellular and molecular processes associated with skin aging and regeneration. *In vitro* models offer a controlled environment to investigate these effects using human-derived cell lines, 3D skin equivalents, or organotypic cultures[22,23,31].

Key biological endpoints include:

- Collagen and elastin synthesis: Quantified via ELISA, immunostaining, or gene expression analysis (e.g., COL1A1, ELN).
- Matrix metalloproteinase (MMP) activity: MMP-1 and MMP-3 are commonly assessed to evaluate extracellular matrix degradation.
- Cell proliferation and viability: Measured using assays such as MTT, Alamar Blue, or BrdU incorporation.
- Oxidative stress modulation: Detection of reactive oxygen species (ROS) and antioxidant enzyme activity (e.g., SOD, catalase).
- Senescence markers: Expression of $p16^{INK4a}$, β-galactosidase activity, and telomere length analysis provides insights into cellular aging.
- Inflammatory cytokine profiling: IL-6, IL-8, and TNF-α levels are monitored to assess immunomodulatory effects.

Advanced techniques such as transcriptomic profiling, proteomics, and high-content imaging further enhance the resolution of efficacy studies. These approaches allow for the identification of subtle changes in cellular behavior and signaling pathways, offering a deeper understanding of the formulation's mechanism of action. The integration of these endpoints into standardized protocols ensures reproducibility and facilitates comparison across different products and studies. Ultimately, biomolecular efficacy serves as a cornerstone for substantiating anti-aging claims and guiding formulation optimization.

Safety and toxicology

Safety assessment is a critical component of preclinical evaluation, ensuring that anti-aging formulations do not elicit adverse biological responses. *In vitro* toxicology provides a reliable and ethically sound alternative to animal testing, aligning with international guidelines such as OECD and ISO standards[24,25].

The main safety endpoints include:

- Cytotoxicity: Assessed via viability assays (MTT, LDH release, Neutral Red uptake) to determine the concentration-dependent effects on cell survival.
- Genotoxicity: Evaluated using comet assay, micronucleus test, or γ-H2AX staining to detect DNA damage and chromosomal instability.
- Skin irritation and sensitization: Reconstructed human epidermis (RHE) models are used to simulate topical exposure and measure inflammatory responses (e.g., IL-1α release).
- Oxidative damage: Quantification of ROS and lipid peroxidation (e.g., MDA levels) helps identify pro-oxidant effects.
- Inflammatory potential: Cytokine profiling (IL-6, TNF-α) and NF-κB activation assays reveal immunostimulatory or immunosuppressive properties.
- Barrier integrity: TEER (transepithelial electrical resistance) and permeability assays assess the impact on skin barrier function, especially for topical formulations.

For injectable products, additional parameters such as hemocompatibility, endotoxin levels, and complement activation may be required. All tests should be conducted under Good Laboratory Practice (GLP) conditions, with appropriate controls and replicates to ensure statistical robustness[25].

The integration of safety data into the formulation development process not only mitigates risk but also supports regulatory compliance and enhances consumer trust[1,26].

Formulation quality

Formulation quality encompasses the physicochemical and functional attributes that determine the stability, bioavailability, and compatibility of anti-aging products. These parameters are essential for ensuring consistent performance, patient safety, and regulatory compliance[26-31].

Key aspects of formulation quality include[6,22-27]:

- Physicochemical stability: Evaluation of pH, viscosity, color, and phase separation under accelerated aging conditions (e.g., temperature, light, humidity). Analytical techniques such as HPLC, DSC, and rheometry are commonly employed.
- Release kinetics: *In vitro* diffusion studies using Franz cells or dialysis membranes assess the rate and extent of active ingredient release from the formulation.
- Bioavailability and penetration: Skin permeation assays using reconstructed human skin or ex vivo explants help determine the depth and distribution of actives.
- Compatibility with biological tissues: Assessment of formulation interaction with skin models, including potential for barrier disruption or irritation.
- Microbiological integrity: Challenge tests and preservative efficacy studies ensure protection against microbial contamination over time.
- Packaging interaction: Studies on extractables and leachables, as well as container closure systems verify that packaging materials do not compromise formulation quality.

For injectable formulations, additional parameters such as sterility, particulate matter, and osmolarity must be rigorously controlled. The use of standardized protocols and validated analytical methods ensures reproducibility and facilitates regulatory approval[22]. Ultimately, formulation quality bridges the gap between laboratory efficacy and real-world application, supporting both product performance and patient satisfaction[1].

The preclinical evaluation parameters used for each evaluation category are summarized in Table 2.

Regulatory and translational considerations

The integration of *in vitro* models into the development pipeline of anti-aging formulations is not only a scientific advancement but also a regulatory necessity. As global frameworks increasingly restrict animal testing, alternative methods have gained formal recognition for safety and efficacy, particularly in the cosmetic and dermatological sectors[1,28].

In the European Union, Regulation (EC) No 1223/2009 prohibits animal testing for cosmetic products and their ingredients, mandating the use of validated *in vitro* assays[29]. The OECD Test Guidelines (e.g., TG 431 for corrosion, TG 439 for irritation, TG 432 for phototoxicity) provide standardized protocols that ensure reproducibility and regulatory acceptance[25].

For medical devices and injectable formulations, the regulatory landscape is more complex. Products are governed by the EU Medical Device Regulation (MDR 2017/745) or by 21 CFR Part 820 in the United States. Although not subject to the same animal testing bans, there is growing emphasis on *in vitro* biocompatibility testing aligned with ISO 10993 standards[30]. These include assessments of cytotoxicity, sensitization, irritation, and systemic toxicity, many of which can be conducted using advanced skin models.

The translational relevance of *in vitro* models depends not only on their biological complexity but also on their ability to predict clinical outcomes. For cosmetic applications, endpoints such as collagen synthesis, antioxidant activity, and epidermal renewal are often sufficient to support product claims. In contrast, medical formulations, particularly injectables, require more stringent assessments, including biocompatibility, immunogenicity, and long-term tissue integration. Recent advances in multi-omics profiling, high-content imaging, and computational modeling have enhanced the predictive power of *in vitro* systems, allowing researchers to correlate molecular changes with clinical endpoints such as wrinkle depth, elasticity, or skin tone improvement[32,33].

One of the main challenges in regulatory acceptance is the lack of harmonization across laboratories and jurisdictions. Variability in cell sources, culture conditions, and assay design can lead to inconsistent results. To address this, initiatives such as the OECD's Guidance Document on *Good In Vitro Method Practices* (GIVIMP) promote standardized procedures and reporting criteria[28].

From a translational standpoint, the predictive value of *in vitro* models depends on their ability to replicate human physiology and correlate with clinical outcomes. This is particularly relevant in anti-aging research, where endpoints like wrinkle reduction or skin radiance are difficult to quantify *in vitro*. Bridging this gap requires the integration of biomarker-based endpoints, multi-omics analyses, and computational modeling to extrapolate laboratory findings to real-world scenarios[26]. As formulations become more complex, incorporating nanocarriers, bioactive peptides, or stem cell-derived factors, regulatory agencies are beginning to acknowledge the potential of organ-on-chip and microphysiological systems. Although formal validation is still underway, these platforms offer unprecedented precision and relevance for safety and efficacy testing[34].

In summary, the regulatory and translational success of *in vitro* models hinges on continued efforts in standardization, validation, and cross-sector collaboration. By aligning scientific innovation with regulatory expectations, these models can accelerate the development of safe, effective, and ethically responsible anti-aging therapies.

Table 2. Preclinical evaluation parameters.

Category	**Key Endpoints**	**Common Analytical Methods**
Biomolecular Efficacy	Collagen, MMPs, ROS, senescence, cytokines	ELISA, PCR, immunostaining, imaging
Safety and Toxicology	Cytotoxicity, genotoxicity, inflammation, TEER	MTT, Comet assay, IL-6 quantification
Formulation Quality	Stability, release, penetration, compatibility	HPLC, Franz cell, rheometry, microbiological tests

Conclusions and future perspectives

The evolution of *in vitro* skin models has redefined the landscape of anti-aging research, offering ethically sound, scientifically robust, and economically viable alternatives to traditional animal testing. These platforms enable precise dissection of cellular and molecular mechanisms underlying skin aging, facilitating the development of targeted interventions with enhanced efficacy and safety profiles.

Despite significant progress, several challenges remain. The complexity of skin aging, driven by intrinsic factors such as genetics and hormonal changes, and extrinsic factors like UV exposure and pollution, requires multifactorial models capable of capturing dynamic interactions across cell types and tissue compartments. Current models, while advanced, often lack components such as immune cells, vascularization, or long-term viability, limiting their translational relevance.

Emerging technologies such as 3D bioprinting, organ-on-chip systems, and integrated multi-omics platforms hold promise for overcoming these limitations. By mimicking the architecture and function of native skin with unprecedented fidelity, these innovations could enable real-time monitoring of aging biomarkers, personalized testing of formulations, and predictive modeling of clinical outcomes. Moreover, the convergence of artificial intelligence and *in vitro* testing is poised to revolutionize data interpretation. Machine learning algorithms can identify subtle patterns in gene expression, protein networks, and morphological changes, accelerating the discovery of novel anti-aging compounds and optimizing formulation strategies.

Looking ahead, the future of anti-aging research lies in the integration of biology, engineering, and computational science. Collaborative efforts across academia, industry, and regulatory bodies will be essential to validate new models, harmonize testing protocols, and ensure regulatory acceptance. As the demand for sustainable and effective skincare solutions grows, *in vitro* models will play a pivotal role in shaping the next generation of dermatological innovation. Ultimately, the transition from empirical formulation to mechanism-driven design marks a paradigm shift, one that places human biology, ethical responsibility, and scientific rigor at the core of anti-aging product development.

As the boundaries between cosmetic and medical aesthetics continue to blur, the development of hybrid products (e.g., cosmeceuticals, bioactive injectables) underscores the need for *in vitro* models that are both mechanistically informative and translationally relevant. Future efforts should focus on validating these models against clinical benchmarks and integrating them into regulatory frameworks that reflect the complexity of modern aesthetic therapies[35,36].

Conflict of interest: The authors declare no conflict of interest.

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