

皮肤抗衰老生物学检测技术进展

Alternative Methods to Assess Anti-Ageing Efficacy

For a safer world

Progress for Skin Anti Ageing Efficacy Bio-test



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内容提要



- 一、化妆品安全和功效测试策略
- 二、皮肤抗衰老生物学机制
- 三、化妆品抗衰老功效评价体外方法
- 四、化妆品抗衰老功效临床测试技术



动物实验 VS 替代实验



动物试验本身的局限：

- 动物实验外推到人存在风险；
- 物种间的差异
- 个体差异
- 环境差异
- 成本效益
- 动物福利和伦理

动物实验在化妆品的适用性：

- 难以定量；
- 无法用于区分配比较和优化；
- 难以用于质控（批次间差异检测）；
- 无法分享现代生物学技术的成果；
- 动物实验报告不能互认（不符合国际标准）
- 皮肤生物学差异
- 动物没有人的功效诉求

化妆品整合测试策略的现状和未来

选择 I 体内实验	选择 II 分层体内实验	选择 III 体外/体内	选择 IV 体外
基于动物生物学	基于动物生物学	人体生物学为主	人体生物学为主
高剂量	高剂量	剂量范围广	剂量范围广
低通量	通量提高	高和中通量	高通量
高成本	低成本	低成本	低成本
耗时	少耗时	少耗时	少耗时
大量使用动物	较少动物	更少动物	几乎不用动物
单一终点	单一终点	毒性通路紊乱	毒性通路紊乱
	少量计算机或体外 筛查	使用计算机筛查	计算机筛查



一、化妆品安全和功效测试策略

testing strategy for cosmetics safety and efficacy

安全评价实验

safety/toxicity test

Eye irritation 眼刺激: HET-CAM+BCOP+RBC
Skin irritation 皮肤刺激: TER+皮肤模型
phototoxicity 光毒性: 3T3 NRU
Skin Sensitization 皮肤过敏: LLNA-BRDU
Percutaneous absorption 皮肤吸收: *in vitro* 扩散池
Genotoxicity testing 遗传试验:
Oral toxicity 经口毒性:
cytotoxicity screen 细胞筛选试验
embryotoxicity 胚胎和发育毒性: EST
Endocrine disrupt 内分泌干扰作用



功能功效实验

Efficacy test

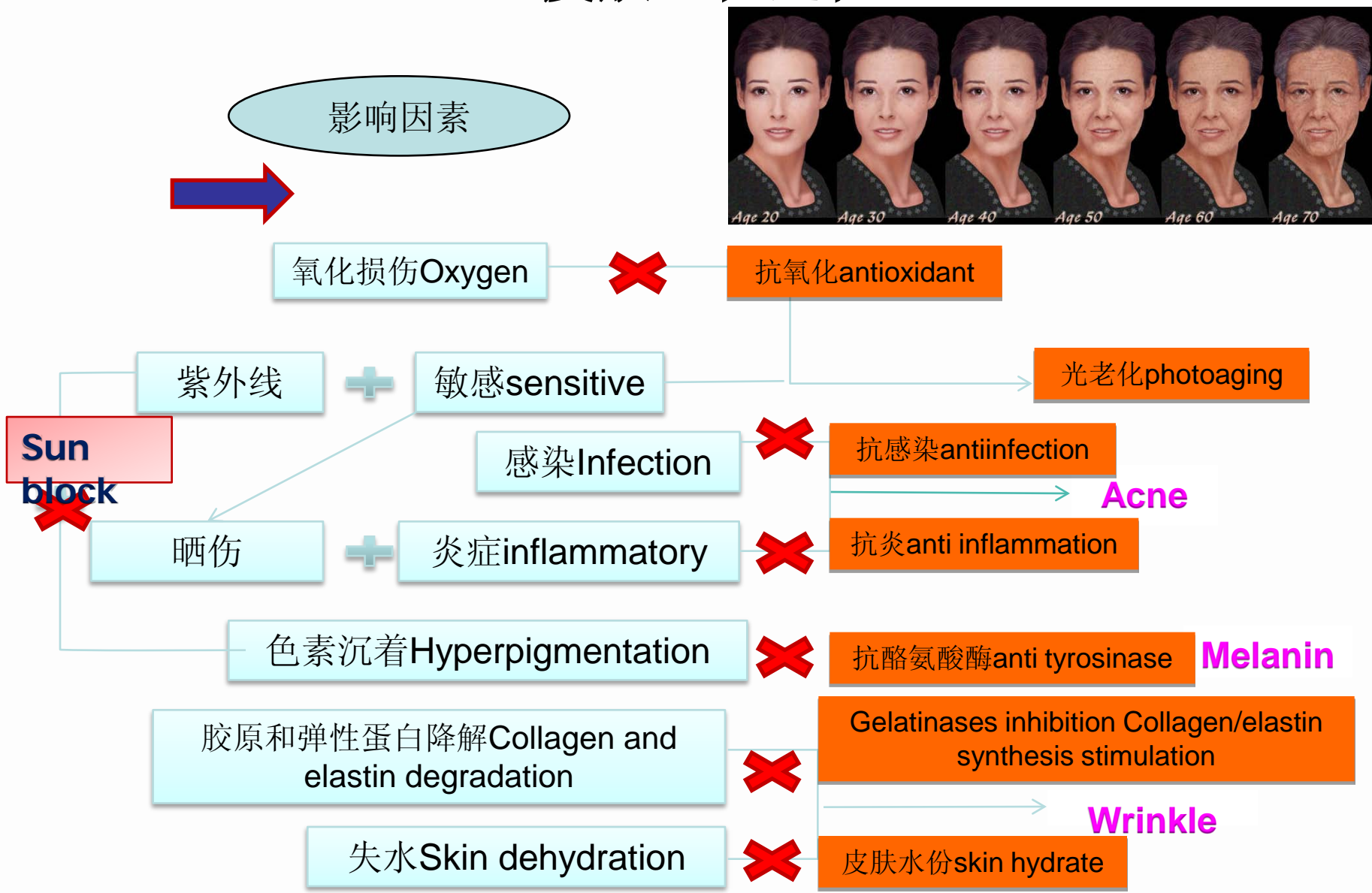
whitening 美白作用
nutrition 营养细胞作用
Anti-oxidant 抗氧化作用
Anti-ageing 抗衰老作用
Anti-inflammation 抗炎作用
vascularization 促进血管生成
Hair growing 生发育发作用
Basic cytomics 基本细胞作用
Patch test 斑贴试验
Photo patch test 光斑贴测试
SPF test instrument assay and human test 防晒指数测试 (体外和人体测试)



适应性和功效测试

Adaptability and Efficacy test

二、皮肤老化过程



光老化 *photoaging*

紫外光对皮肤的危害

UVB急性反应引起皮肤表面的红斑、炎症和晒黑。

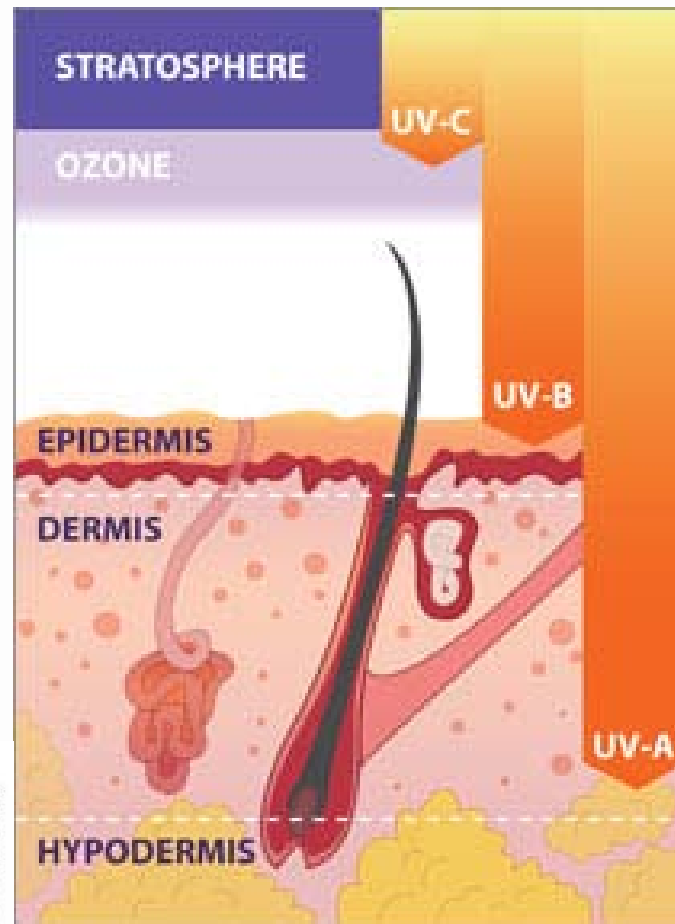
UVA慢性反应引起皮肤的光老化，真皮基质成分的变化。

光过敏和光敏性皮肤病。

UV Light



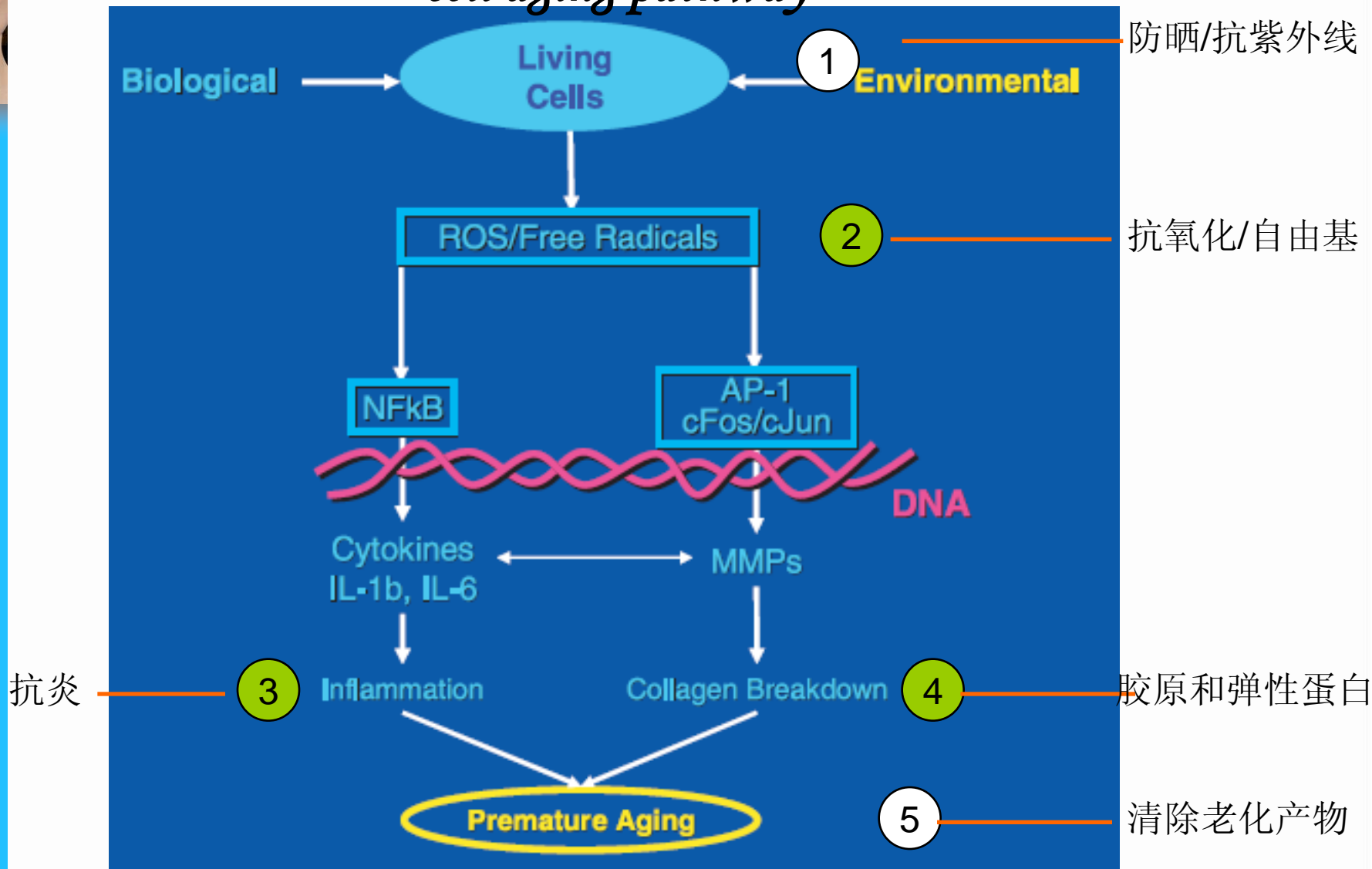
Before





皮肤细胞老化过程

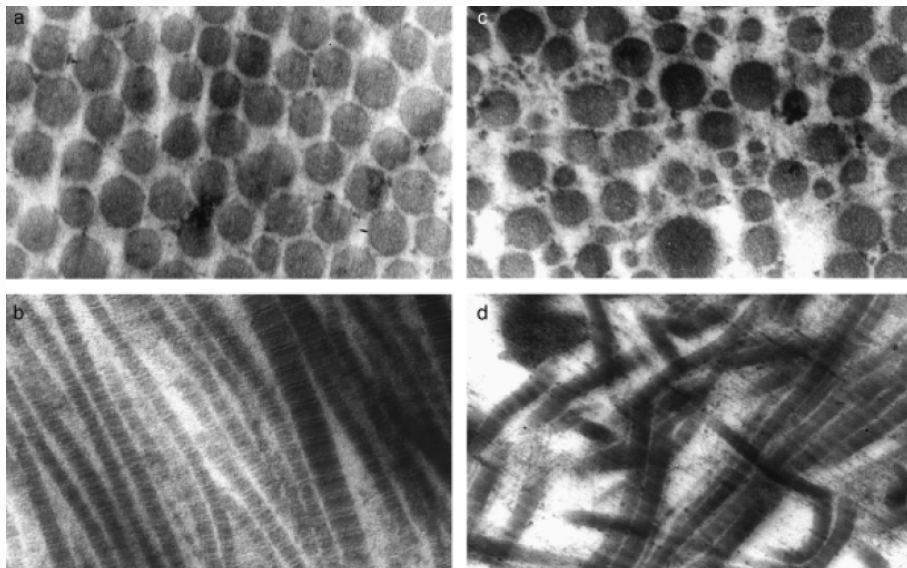
cell aging pathway



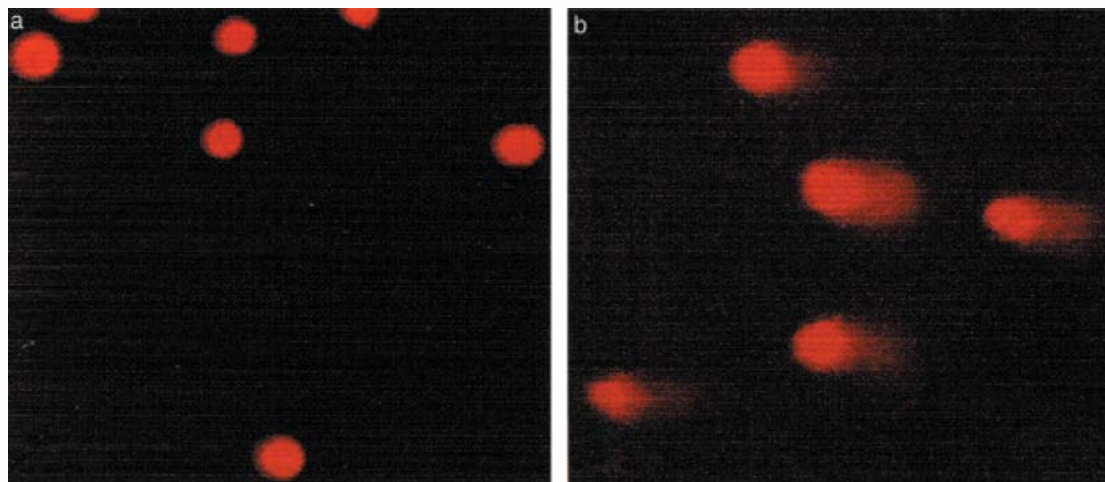
三、化妆品抗衰老功效评价体外方法

1、防晒 *Sunscreen*

体外细胞模型研究-原理



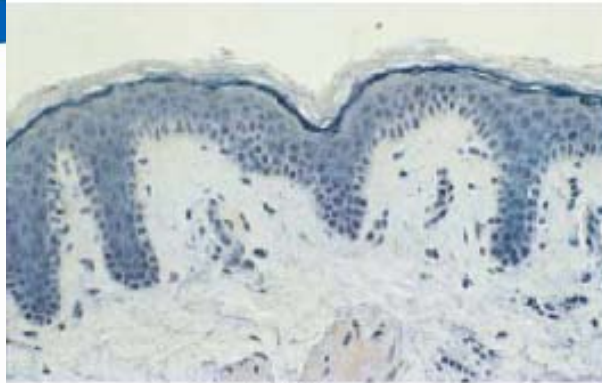
透射电镜观察



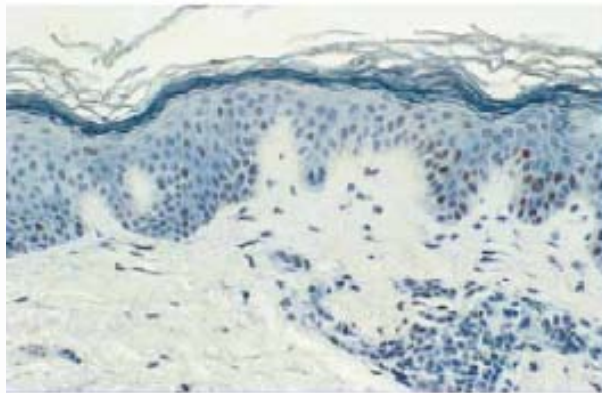
角质细胞彗星试验

体外细胞模型研究

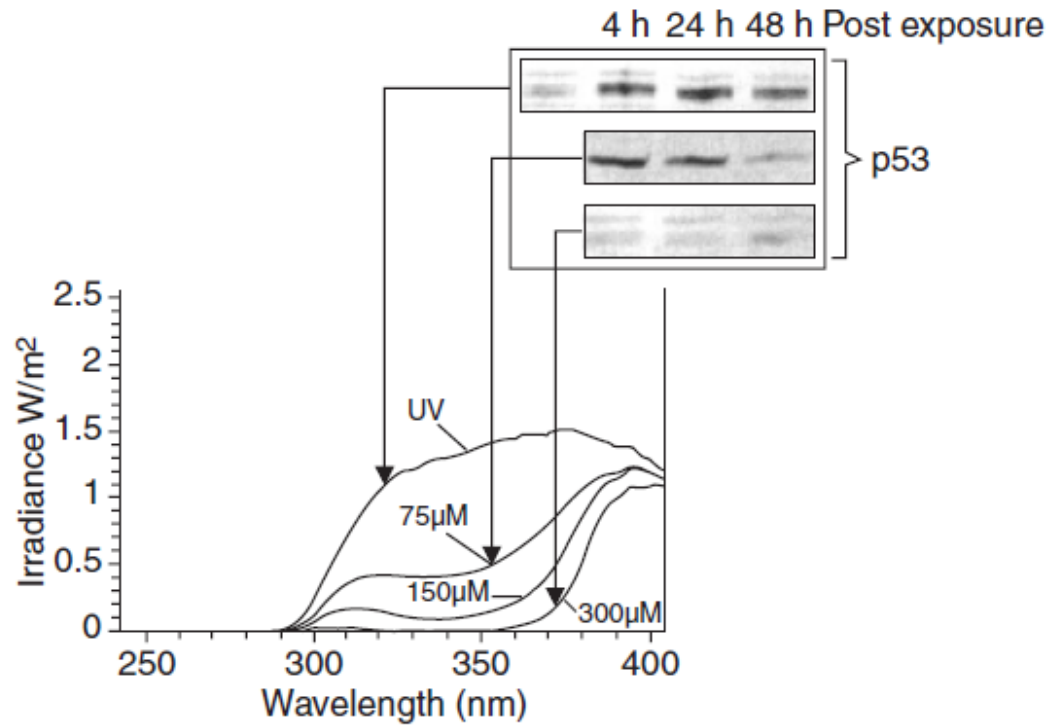
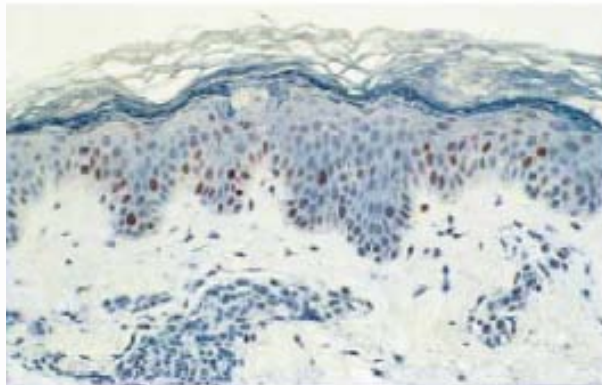
—适用于原料筛查



Control



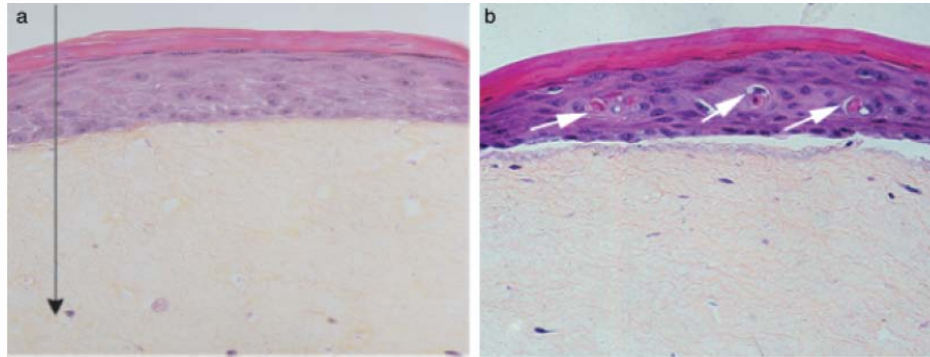
Sunscreen A



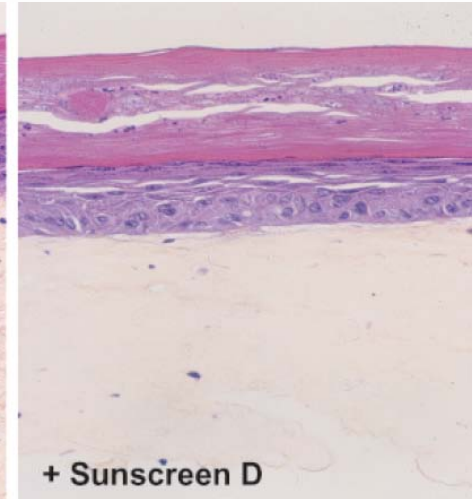
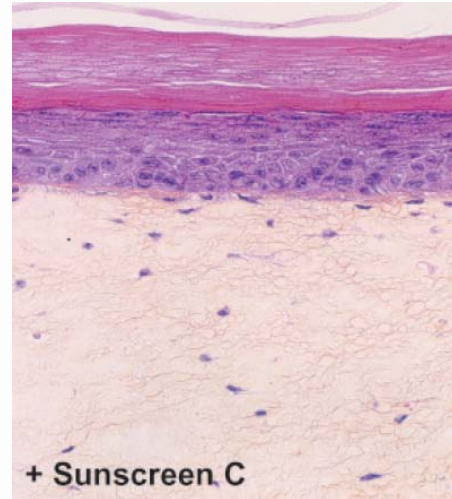
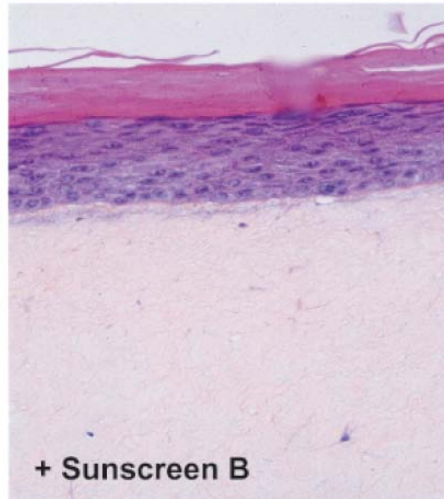
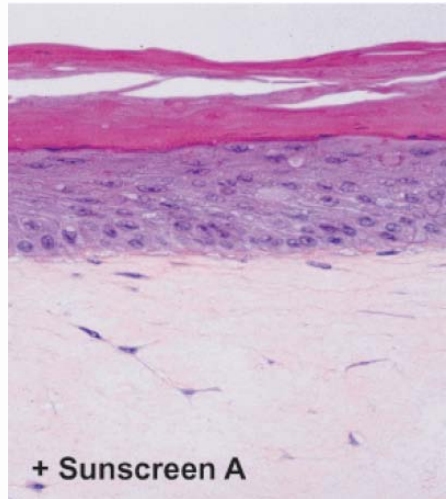
角质细胞核P53水平测试

经防晒剂处理后的细胞再经UV照射，P53水平下降

体外皮肤模型研究—适用于配方和产品设计



UV损伤的皮肤模型



不同防晒剂防晒效果比较

剂量: 40J/cm²

1、防晒 *Sunscreen*

测试目的：原料？确认？配方？产品？

急性暴露？长期暴露？

UVA？UVB？UVA+UVB？

方法选择：适用性和科学性

测试系统：皮肤成纤维细胞和角质细胞？原代？细胞系？

离体皮肤、重建表皮、重建皮肤

检测指标：DNA损伤（CPDs, 8-oxo-dG），应激反应（p53, HSPs）、

凋亡（caspase-3, 晒伤细胞, DNA片断）

炎症（LC细胞迁移、细胞因子）

结果描述：

阳性对照，阴性对照

2、抗氧化与自由基损伤

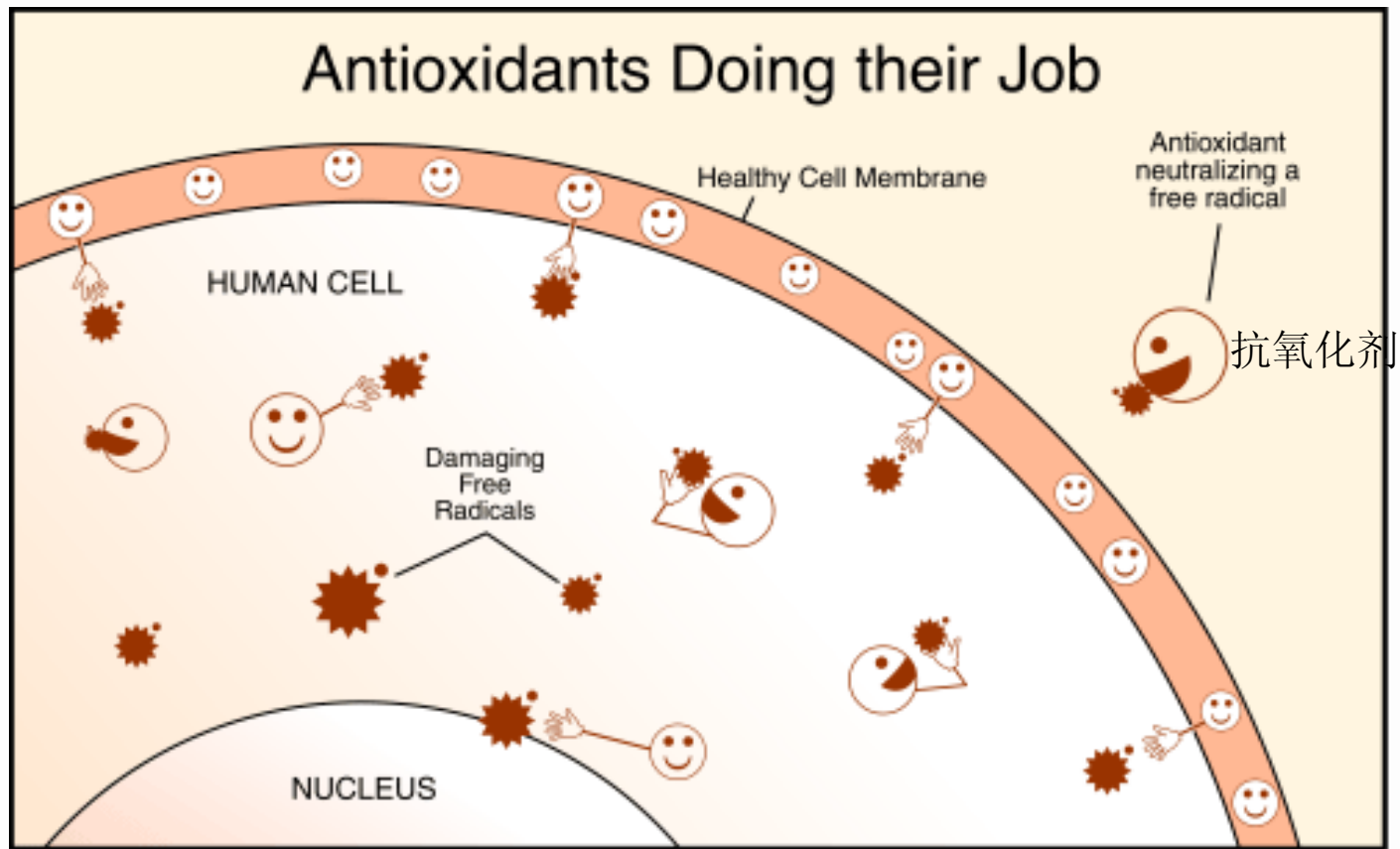
Antioxidants and Free Radical Damage



*"Electromagnetic pollution (EMF) may be the **most significant form of pollution** human activity has produced in this century!"*

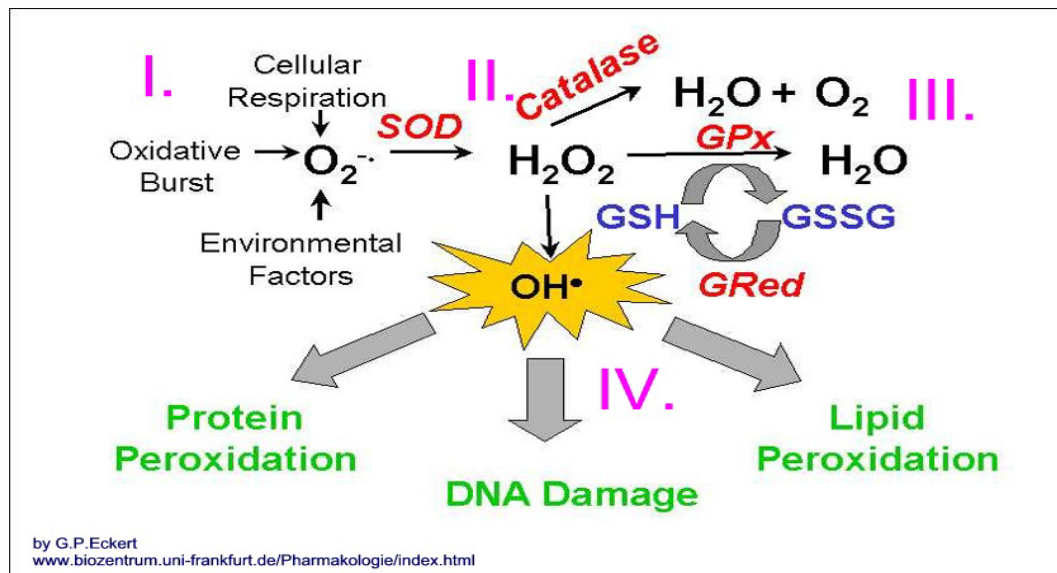
2、抗氧化与自由基损伤

Antioxidants and Free Radical Damage



抗氧化测试 *Antioxidant assay*

细胞氧化应激
Cellular
Oxidative stress



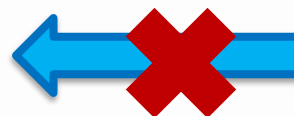
氧化酶oxidative enzyme ↑

- Superoxide
- Glutathione peroxidase
- Catalase

抗氧化能力下降
Antioxidant capacity ↓

Antioxidants in cosmeceutical

- ☐ Vitamin C
- ☐ Vitamin E
- ☐ Glutathione
- ☐ Carotenoids
- ☐ Other



抗氧化活性检测：化学法

Antioxidant assay

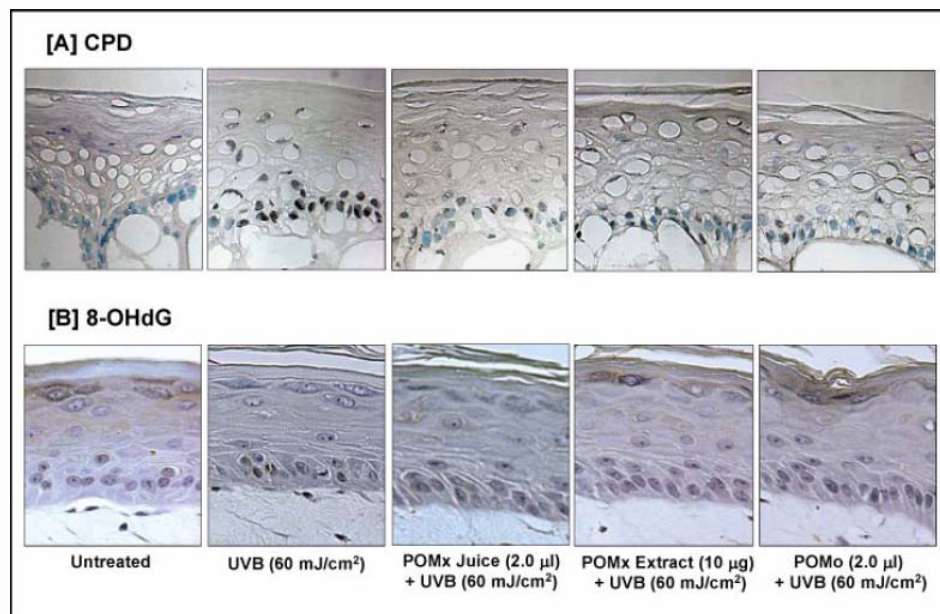
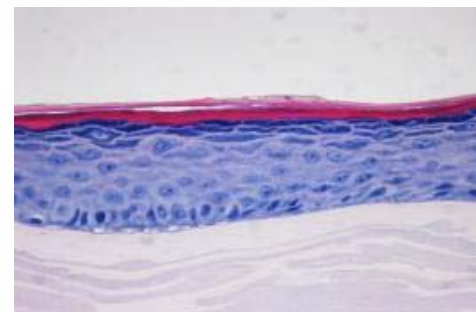
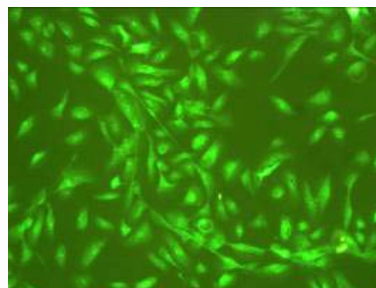
- **DPPH Method**
- **FRAP Method**
- **ABTS Method**
- **ORAC Method**

适用于原料筛查

局限：无生物系统参与

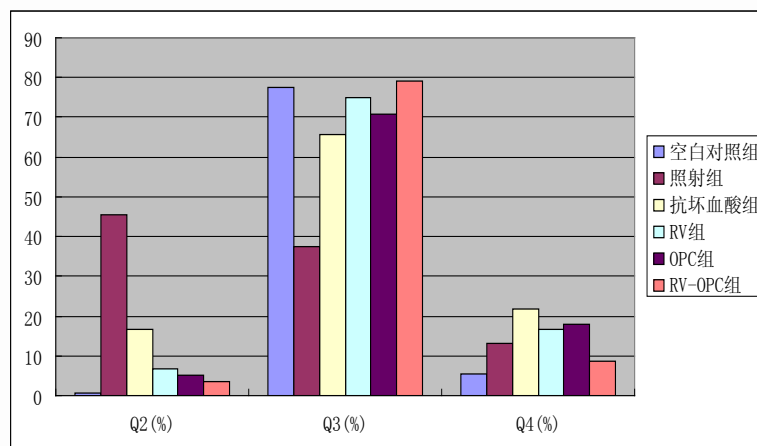
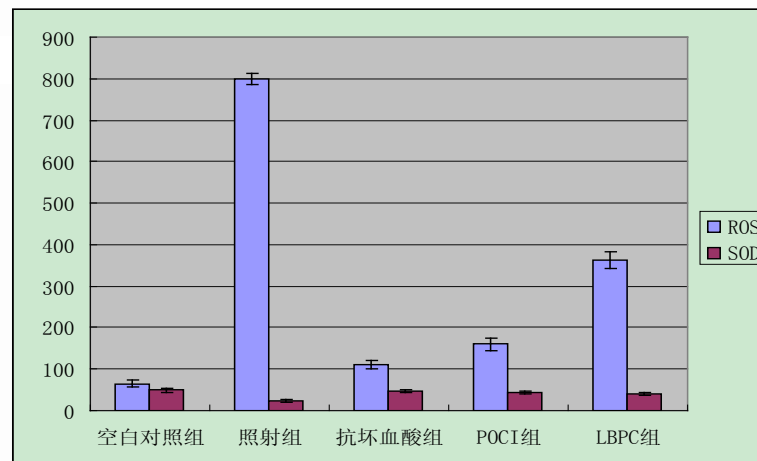
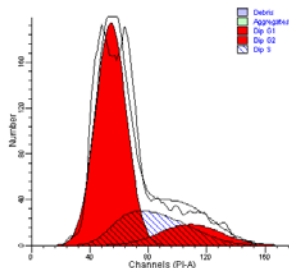
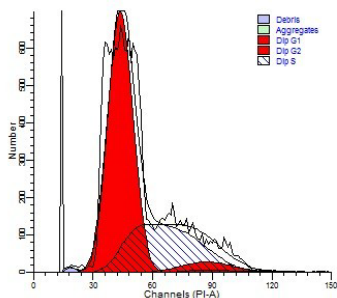
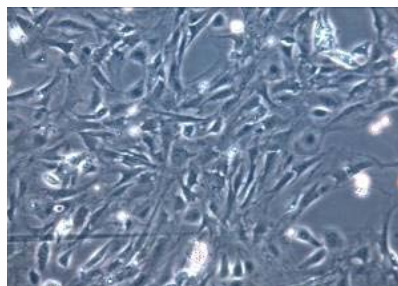
细胞水平抗氧化测试

- 细胞系统：人角质细胞和成纤维细胞，两种细胞共培养系统
- 皮肤模型：单层和全层颇佳模型
- 臭氧、UVA/UVB等氧化损伤
- 检测多种指标：细胞周期、凋亡、直接检测自由基、酶、氧化产物、DNA损伤（CPD、8-OHdG）等；



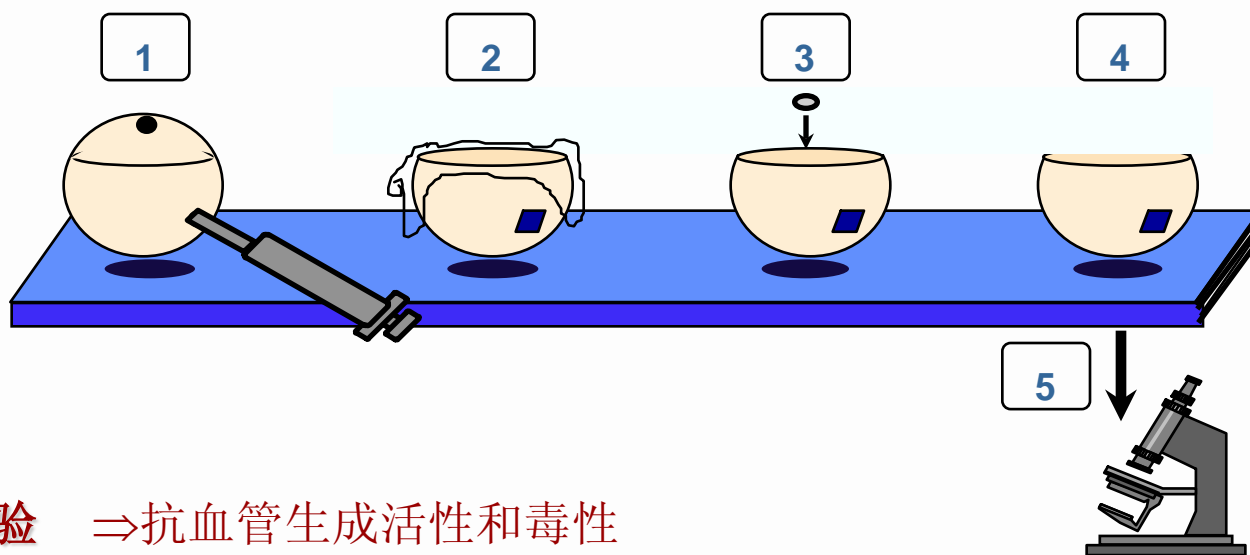
细胞水平抗氧化测试

- 适用于化妆品原料筛查;
- 配方优化;
- 用于机制研究;
- 细胞之间的相互作用;
- 损伤和预防机制



器官水平抗氧化测试

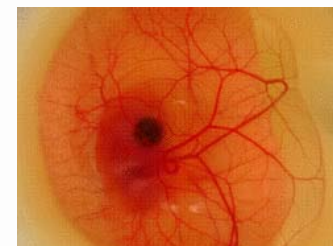
——绒毛尿囊膜试验 antioxidant-CAM assay





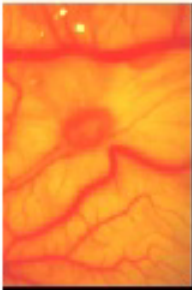
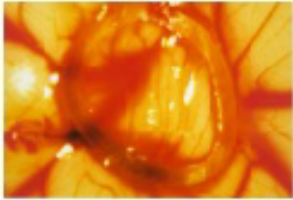
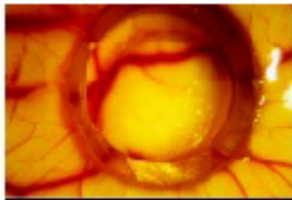
▪**CAM-试验** ⇒ 抗血管生成活性和毒性
Antiangiogenic activity and toxicity

▪**HET-CAM-试验** ⇒ 抗炎症活性
Anti- inflammatory activity

▪**Ring-HET-CAM-试验** ⇒ 抗氧化活性Antioxidant activity.



绒毛尿囊膜试验 CAM assay

<p>I. CAM-Assay</p>  <ul style="list-style-type: none"> - no antiangiogenic effect - no membran irritation - no toxic effect 	<p>抗血管刺激作用 Anti-angiogenic Irritant Toxicity</p>
<p>II. HET-CAM-Assay</p> <div style="display: flex; justify-content: space-around;"> <div style="text-align: center;">  <p>inflammation caused by SDS (50µg/pellet)</p> </div> <div style="text-align: center;">  <p>antiinflammatory effect of PE (250 µg/pellet) Viitamin C: no effect</p> </div> </div>	<p>抗炎Anti-inflammatory</p>
<p>III. Ring-HET-CAM-Assay</p> <div style="display: flex; justify-content: space-around;"> <div style="text-align: center;">  <p>CAM-irritation caused by Fenton-reagent combined withVitamin C (50µg/pellet)</p> </div> <div style="text-align: center;">  <p>PE (250µg/Pellet) caused no CAM- irritation combined with Fenton-reagent</p> </div> </div>	<p>抗氧化Antioxidant Pro-oxidative</p>

抗氧化活性检测

测试目的：原料？确认？配方？产品？

方法选择：适用性和科学性

测试系统：皮肤细胞其它体细胞？

结果描述：

阳性对照，阴性对照，

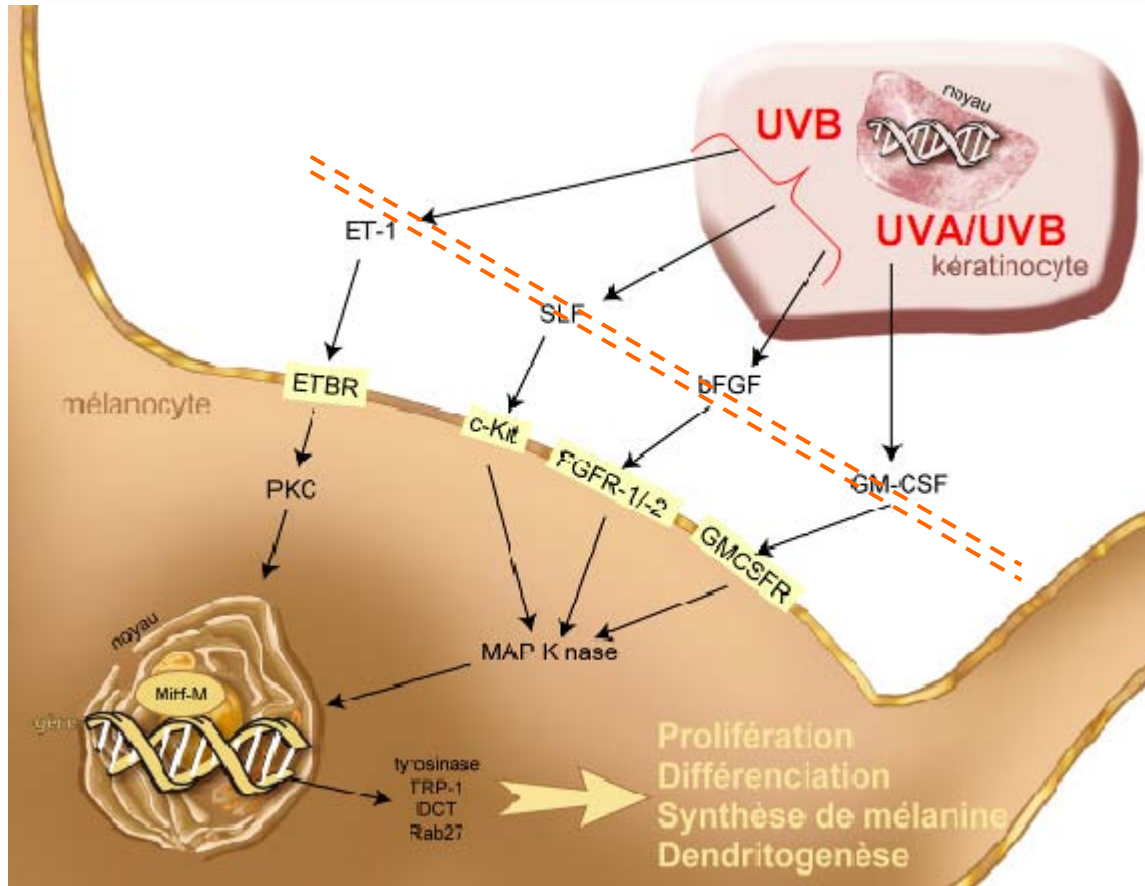
- **Tocopheryl Acetate (Vitamin E):** A powerful antioxidant and helps to protect skin from harmful effects of free radicals and other radiations - increase the moisture of the skin's epidermal layer and thereby improve surface relief.
- **Retinyl Palmitate (Vitamin A):** used to increase collagen production - a wrinkle fighter and an exfoliant - well known to normalize skin and make it supple - will also help to keep skin soft and plump making it younger looking - clinical results have shown that it will increase collagen, skin thickness, and elasticity, significantly increasing skin composition.
- **Ascorbic Acid (Vitamin C):** the only antioxidant that is proven to stimulate the synthesis of collagen, which is essential since the body's natural collagen production decreases with age - collagen is responsible for warding off wrinkles and fine lines - studies have shown that vitamin C helps to minimize fine lines, scars and wrinkles.

抗氧化活性成份 *Active Ingredients*

- **巴西紫莓提取物Acai Berry Extract:** helps kick-start collagen synthesis - provides free-radical protection - eliminates toxins for a healthier complexion - **softens the appearance of fine lines and wrinkles** - **improves elasticity** - renews skin's natural glow.
- **红石榴提取物Pomegranate Extract**- contains extremely high levels of polyphenols; powerful antioxidants that **help stabilize free radicals** which cause premature aging - new research has shown that pomegranate may be one of the most naturally potent wrinkle-fighters to be used in effective skincare today.
- **葡萄籽提取物Grape Seed Extract** - a very powerful antioxidant - **contains proanthocyanidins that strengthen blood vessels** and help to improve circulation - this is said to be 20 times more powerful than Vitamin C and 50 times more powerful than Vitamin E and even more powerful than Beta Carotene. It is an extended anti oxidant that stays in your blood stream for 3 full days.
- **没药醇和生姜根提取物Bisabolol and Ginger Root Extract:** (an active ingredient of Chamomile plant) is known for its powerful **anti-inflammatory and soothing benefits to skin** - when combined with Ginger root extract, it provides extremely powerful skin balancing and rejuvenation benefits - its composite effects result in giving skin a much healthier, less irritated and youthful appearance

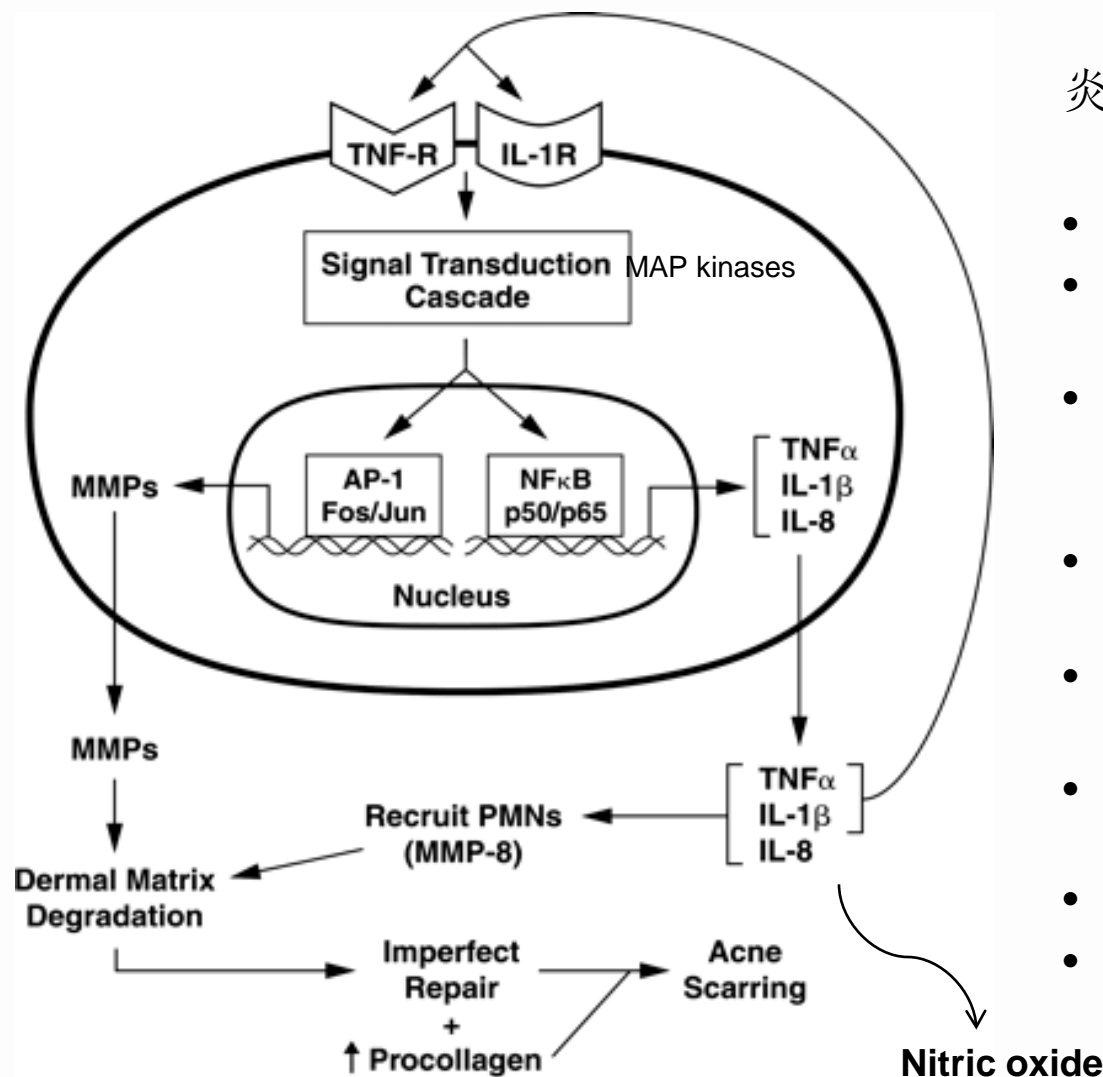
3. 抗炎试验 *Anti inflammation assay*

抑制/中和化学介质: GM-CSF、BFGF、SLF、ET-1



Inhibition of ET-1 production

3. 抗炎试验 *Anti inflammation assay*



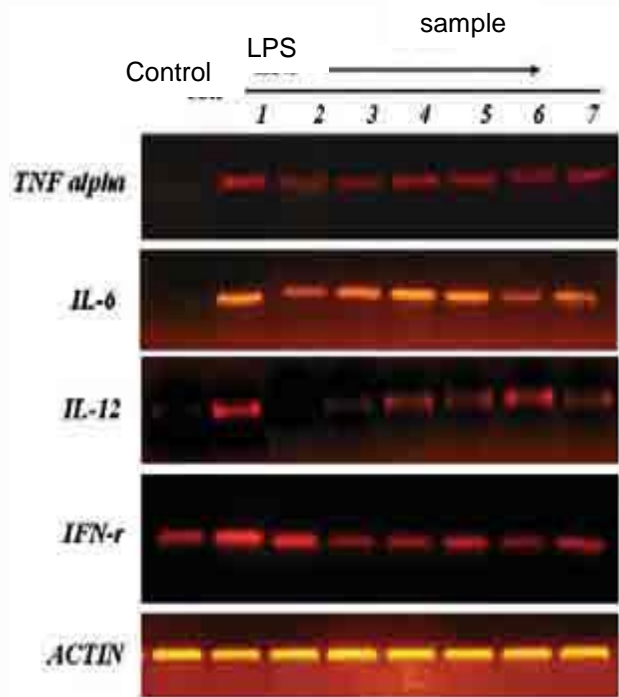
炎症与皮肤痤疮的关系

- 炎性痤疮, NF-κB信号通路激活
- 炎症因子基因激活 (如TNF-α和 IL-1β) .
- TNF-α和IL-1β 激发次级细胞因子增生, 如IL-8, 启动 MAP 激酶活化产生刺激作用的 AP-1
- AP-1-驱动基质金属蛋白酶 (MMPs) 产生
- 随着胶原酶和弹性酶到达作用位点, 合成胶原蛋白和弹性蛋白
- 不完全的修复导致色素沉着、表面粗糙等难以描绘的缺陷.
- 前胶原蛋白持续合成
- 粉刺和疤痕形成

3. 抗炎试验 *Anti inflammation assay*

抑制前炎症因子
TNF- α , IFN- γ , and IL-1 β

转录 (RT-PCR)



抑制炎症通路
Translocation of NFkB p65
Subunit in Nucleus

转录 (western blot)



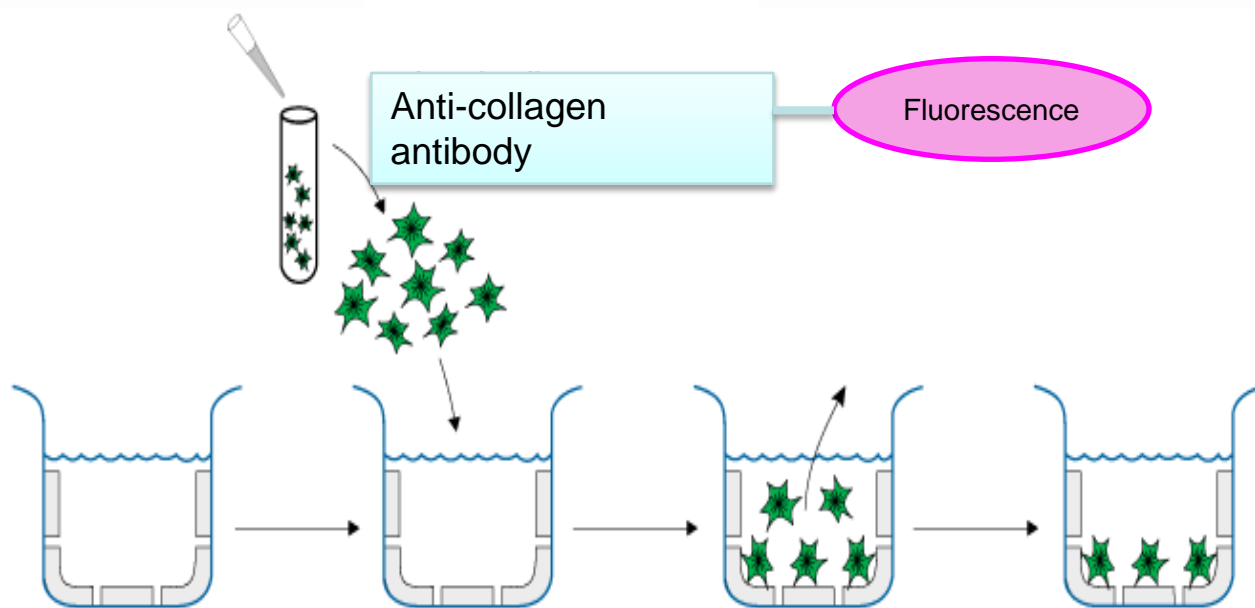
4. 胶原和弹性蛋白合成

Collagen/elastin synthesis

激发胶原蛋白合成

免疫荧光技术

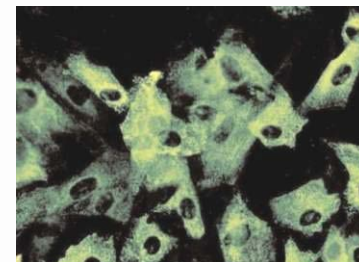
- 单克隆抗胶原蛋白抗体，结合荧光标志物



Blank



Test compound

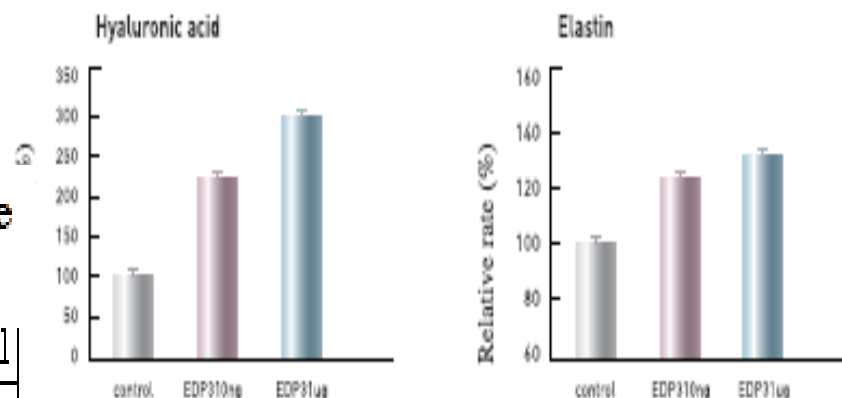
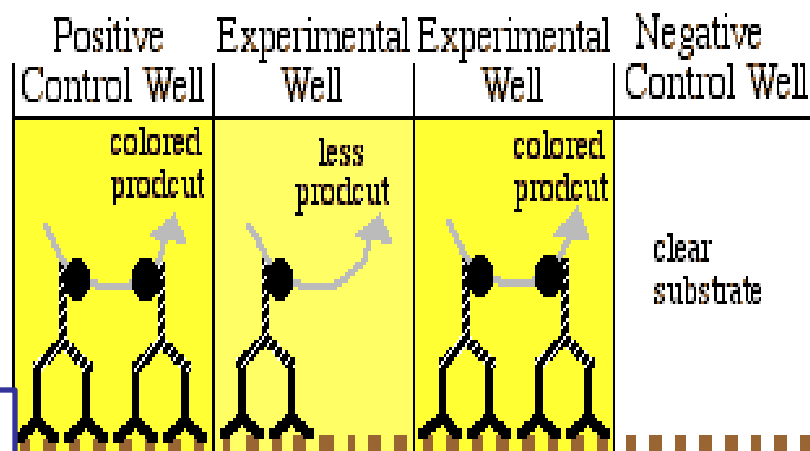


激发透明质酸和弹性蛋白合成

ELISA方法

原理：检测特殊蛋白（透明质酸和弹性蛋白）在成纤维细胞中的含量是否增加，3个浓度的受试物处理细胞，96孔板培养，孔底包被抗体，用于结合所测定的蛋白，实现高通量筛查。

Side View of 4 Wells from a 96-well Plate



抑制基质金属蛋白酶

基质金属蛋白酶家族的作用是降解细胞外基质蛋白

MMP-2和MMP-9（白明胶酶A和B）的主要作用是降解胶原蛋白和弹性蛋白

角质细胞和成纤维细胞共培养

Human keratinocyte : treated with **test compound** at 3 different concentration

Human fibroblast in multiwell trays

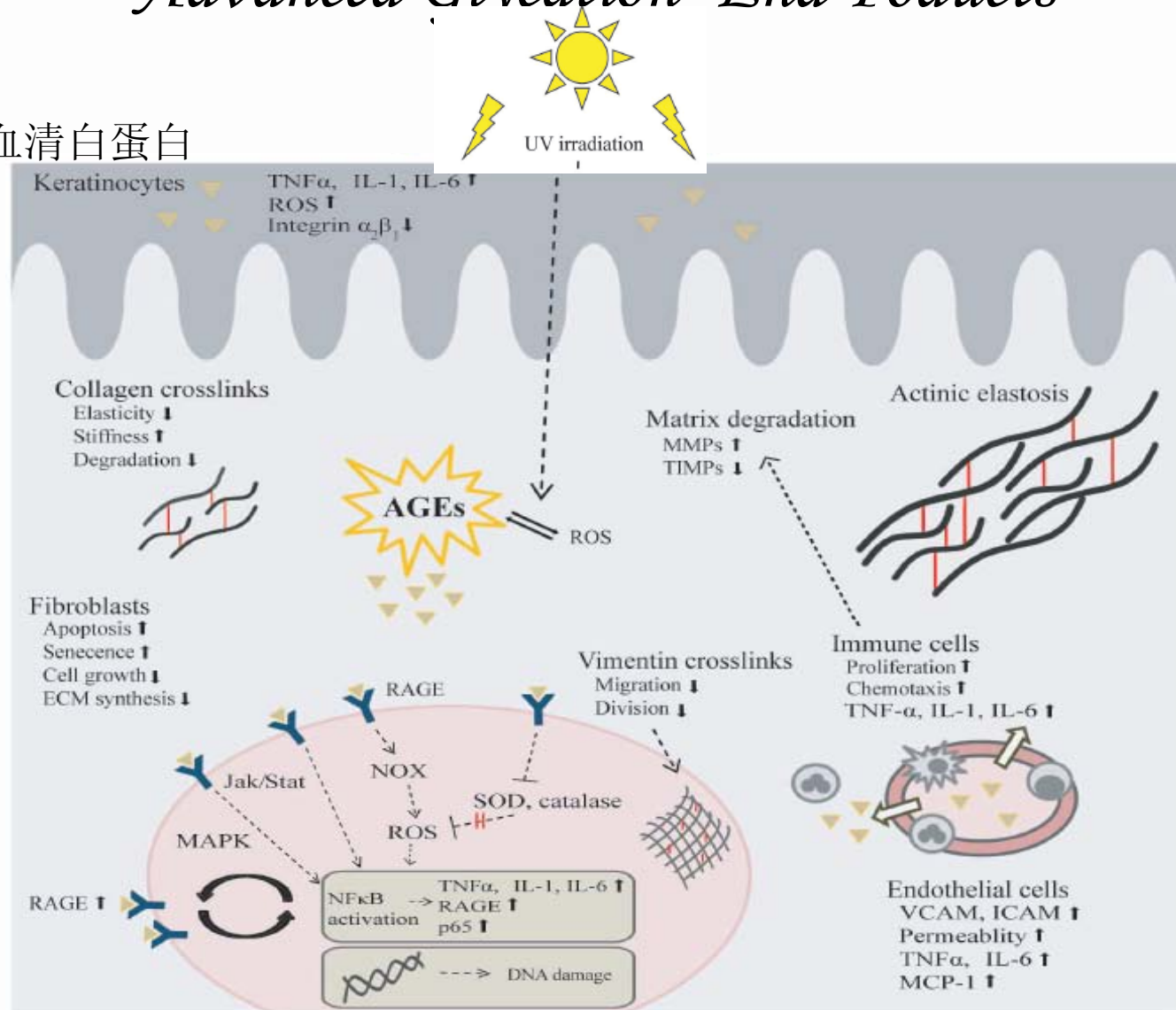
UV irradiation

To evaluate the expression of MMP and TIMP1 (PT / PCR)

MMP和TIMP1表达水平的下降，表明测试样品具有抗光老化作用

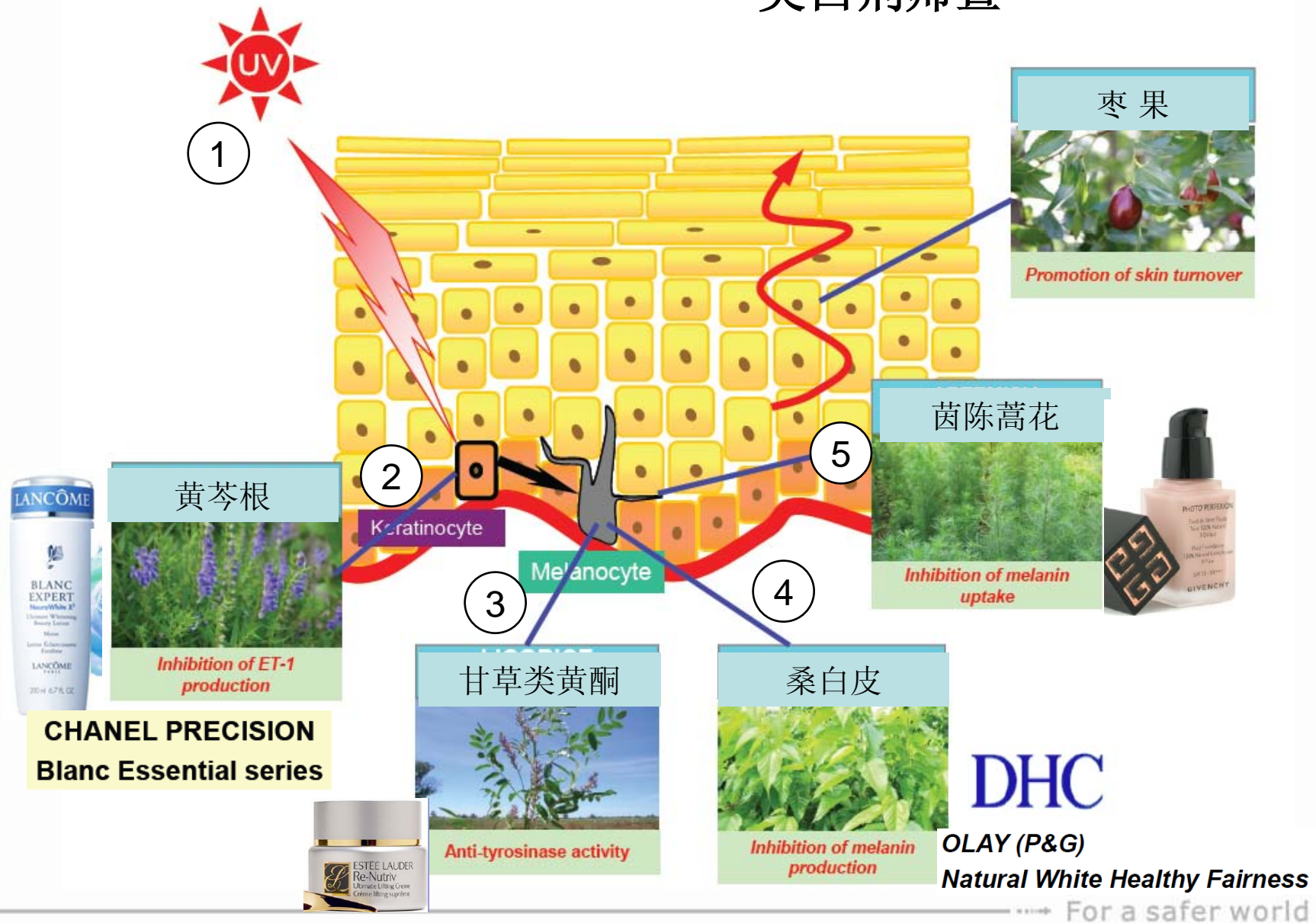
5. 抑制糖基蛋白终物 *Advanced Glycation End Products*

体外抑制HAS人血清白蛋白
脱氧葡萄糖醛酮
戊糖素
羧甲基赖氨酸



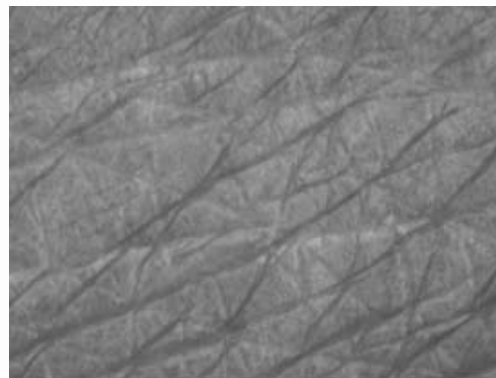
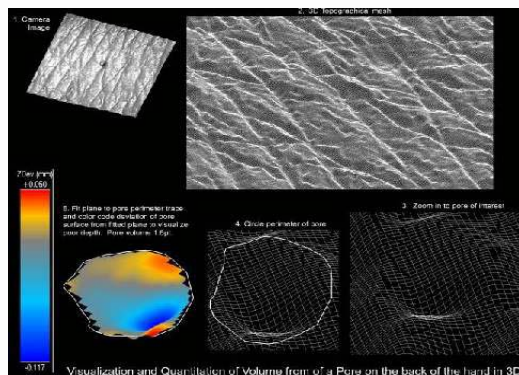
紫外线光损伤的生物学

-----美白剂筛查



四、化妆品抗衰老功效临床测试技术

- 1、保湿：水份含量和水份流失
- 2、皱纹：
- 3、皮肤弹性
- 4、皮肤光泽度
- 5、全脸分析/皮肤图像分析



四、化妆品抗衰老功效临床测试技术

现有临床功效评估的不足

- 1、临床实验的标准化；
- 2、分析软件的2次开发：
- 3、数据解析和挖掘；
- 4、数据共享不够
- 5、原料筛查与临床测试组合策略



抗皮肤衰老化妆品功效测试策略

原料功能筛查

防晒:
抗氧化/自由基:
抗炎:
促胶原/弹性蛋白合成:
清除有害产物:
修复:
营养皮肤细胞:
保湿:
.....



安全性替代试验

眼刺激: HET-
CAM+BCOP+RBC
皮肤刺激: TER+皮肤模型
光毒性: 3T3 NRU
皮肤过敏: LLNA-BRDU
皮肤吸收: *in vitro*扩散池
.....

临床功效实验

皮肤水份测试
皮肤水份流失测试
全脸分析和图像解析
显微图像分析
皮肤弹性测试
皮肤皱纹测试
斑贴试验
体外防晒指数测试
体内防晒指数测试



化妆品安全和功效检测技术咨询

1 替代方法共识平台

Chinese Center for Alternatives Research & Evaluation (CCARE)

<http://www.vitrotox.com>

2. 学术专著

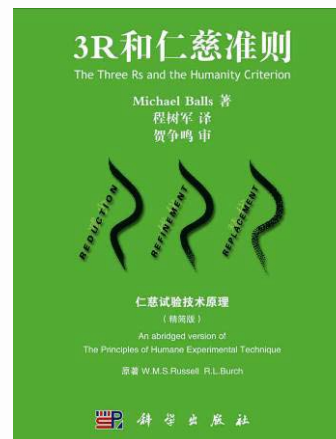
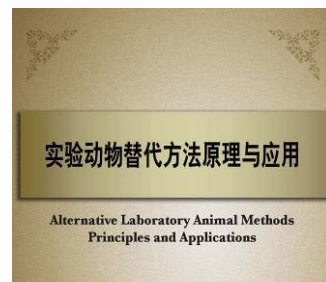
实验动物替代方法原理与应用. 科学出版社, 2006-2010

Alternatives Laboratory Animal Methods principle and Application, Science Press, 2010

动物试验替代方法指南, 科学出版社, 2010-2014

Guideline for Alternative Animal Testing Methods, Science press, 2014

3. 3R和仁慈准则 The Three Rs and the Humanity Criterion



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2010~2014



"This is ridiculous. We can't test the products on animals, but should we test them on human beings instead?"

KINOPOLITE, A RUSSIAN COSMETIC MANUFACTURER FOR SKINCARE IN RUSSIA

"Animals are animals and human beings are human beings. The results of tests conducted on animals do not necessarily reflect the effects... on human beings."

ZHANG QUANCHEN, SENIOR SCIENTIST AND PROFESSOR IN THE INSTITUTE FOR VITRO SCIENCES IN THE UNITED STATES

Testing: A humane move, or a cosmetic change?

FROM PAGE 4

"It will be a very good thing for domestic manufacturers in China, because it will enable them to sell their products in the U.S. as long as no other testing has been done for their products since March 11, 2013," said Troy Smith, director of the Research and Testing Group at the Center for the United States Global Human Society International.

However, experts said China still has a long way to go before it can match international standards in the use of in vitro methods — tests conducted on cells grown in petri dishes or test tubes — and other alternative methods.

"It is not simply a shift in testing methods from animals to in vitro methods. There is still much more work to be done at the infrastructure level and to technological standards," said Cheng Shupin, director of the Technology Center at the Guangdong Institute of Quality and Technical Center.

Cheng said a trial ban on animal testing would be premature, in part because in vitro testing procedures are very demanding. "We are not ready to replace certain testing methods. We can only try to reduce the use of animals," he said.

Zhang Quanchen, a senior scientist and program manager with the Institute for Vitro Sciences in the United States, said it could take a long time for Chinese laboratories to change the way they conduct research. "Initially, the cosmetics industry has to undergo initial growth, not in terms of testing methods and technology, it will take time," he said.

According to Cheng, only two Chinese cosmetics companies have the ability to produce 100,000 units of in vitro testing methods, which allow cells to grow and interact with the environment in all three directions — for testing, while in the U.S. there is a wide industry chain. "After all, the priority is to guarantee the safety of consumers," he said.

A late start

Compared with European countries and the United States, China had a late start in the development of in vitro methods, and the cosmetics industry only started researching the procedures about a decade ago, according to Cheng, who said that low scientific confidence research into alternative testing is the early part of the industry.

"The reason why these methods have attracted the attention of a growing number of researchers and scientists is that research is slowly picking up. The companies with Western cosmetics brands still call it a 'huge gap'."

"The reduction of the last generation of cosmetics products has raised concerns among Chinese consumers. A rapid networking on Weibo, a social networking site, showed that consumers were of the opinion that all in vitro testing would compromise product safety. This is ridiculous. We can't test the products on animals, but should we test them on human beings instead?"

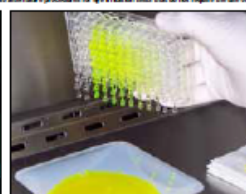
Some consumers identified by the Chinese media as "cosmetic cowards" who said the change would harm their health. "Our products will become less safe," said one consumer. "We are not sure if the new testing methods will be as effective as the old ones," said another.

Cheng Shupin said the new testing methods would require a large amount of investment, which would be a challenge for some small enterprises. "We will have some initial testing centers and centers for research and development," he said.

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Researchers at a training session learn alternative procedures for eye irritation tests that do not require the use of live animals.



Researchers at the Institute for Vitro Sciences in the United States conduct a pharmacology test on a cosmetic product using the new test methods.

There will be some initial test centers for Chinese cosmetics companies to help them learn the craft to conduct in vitro tests. However, the use of validated alternative tests will be both cheaper and faster than using animals, so the companies will benefit greatly from this saving in test costs.

JUDY MACARTHUR CLARK, CEO OF THE ANIMAL RESEARCH REGULATORY CENTER AT THE UNITED STATES GLOBAL HUMAN SOCIETY

There is a need for a national coordination mechanism to popularize alternative testing methods.

CHENG SHUPIN, DIRECTOR OF THE TECHNOLOGY CENTER AT THE GUANGDONG INSTITUTE OF QUALITY AND TECHNICAL CENTER

AT THE MEETING OF THE GUANGDONG INSTITUTE OF QUALITY AND TECHNICAL CENTER

There is a need for a national coordination mechanism to popularize alternative testing methods. "There is a need for a national coordination mechanism to popularize alternative testing methods," he said. "There is a need for a national coordination mechanism to popularize alternative testing methods," he said.

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compensation directly to the Organization for Economic Cooperation and Development, which stipulate that all cosmetic products must be subjected to "animal testing" before being sold in the U.S. market, including those that are already approved in other countries. The OECD stipulations, which include a range of alternative procedures, have allowed the firms to use the in vitro methods to meet the requirements of the U.S. market. "Animals are animals and human beings are human beings. The results of tests conducted on animals do not necessarily reflect the effects of the products on human beings," said Zhang from the Institute for Vitro Sciences in the United States. "Zhang said the company of eye irritation tests, traditionally conducted on rabbits, to replace his case. "The eye irritation test is a very old test, it meets the eye of rabbits but only for the eye, while human beings have 12 layers. How can you expect one test to predict the safety of human beings?" he said.

Some cosmetics companies said they have long been troubled by the use of animals in testing. "We have the same batch of products in three test agencies under different government departments and the results are completely different. As we don't know which result to trust," said Chang Lian, captain and co-owner manager with Bioscience Inc., a Changsheng-based cosmetics company.

Chang said the company has always been a good investor in in vitro products because they often provide new insights into products, and some alternative methods, including in vitro, have advantages over traditional tests, such as the shorter time needed to yield results.

However, the biggest advantage lies in the fact that in vitro tests are designed to simulate human skin, and the results are more accurate. "The source of error is a problem. For instance, one of the in vitro methods involves the use of human skin cells, but in certain cases, they are not representative of human skin," said Chang Lian, a researcher from the Shanghai Institute of Food and Drug Control, said the institute has sent members to the U.S. to train in the use of in vitro methods, and he also purchased the necessary equipment and built the laboratory for their implementation.

However, despite the acceptance of the need to keep abreast of technological changes, the institute still has a number of problems, he said. "The source of error is a problem. For instance, one of the in vitro methods involves the use of human skin cells, but in certain cases, they are not representative of human skin," said Chang Lian, a researcher from the Shanghai Institute of Food and Drug Control, said the institute has sent members to the U.S. to train in the use of in vitro methods, and he also purchased the necessary equipment and built the laboratory for their implementation.

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一、会议主题 替代方法的标准化验证与新技术应用

二、会议主办：

三、会议时间：2014年11月12~15日。

四、会议地点：成都高新西区西芯大道 3号，通用（GE）中国创新中心会议室。

五、会议官网：

替代方法研究评价中心，<http://www.vitrotox.com>

欢迎参加第三届替代方法研讨会

六、会议内容

4个专题

专题1：替代试验方法的研发与国际进展

专题2：皮肤模型替代技术的研究验证与应用

专题3：替代方法在化妆品安全评价中的应用

专题4：替代方法开发中新技术应用

2个讨论

讨论1：皮肤模型的研究与验证模式探讨

讨论1：新毒理学替代试验方法的标准化

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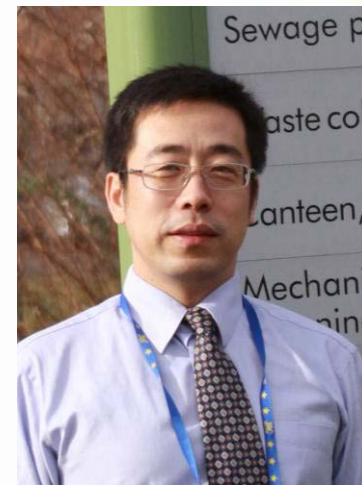
二、丛书总编和召集人

三、各册简介（暂定）

1. 《化妆品法规管理与风险评估指南》
2. 《化妆品安全评价与替代技术指南》
3. 《化妆品健康环境影响与化学分析》
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程树军 博士 研究员

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