REVIEW

Recent Trends of Alternative Methods to Phototoxicity Testing in Japan

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Abstract

Several *in vitro* phototoxicity methods have been developed to assess the phototoxic potential of substances. These can be classified into two groups: the methods for screening purposes and tests focusing on the specific mechanisms of phototoxic reactions.

Among these methods the 3T3 Neutral Red uptake phototoxicity test (3T3 NRU PT) was accepted as an established alternative method by ECVAM. In 2000, the EU Commission officially accepted 3T3 NRU PT for classification and labeling of chemicals to assess their acute phototoxic potential. In 2004, 3T3 NRU PT was adopted as an OECD guideline for chemicals (OECD Test Guideline TG432).

This paper will describe the peer review of 3T3 NRU PT in Japan and the validation study of a new battery system proposed by Japanese industry. The paper also discusses a few recent studies using 3T3 NRU PT.

***Key word**: in vitro phototoxicity, 3T3NRU phototoxicity test, validation study, Yeast-RBC assay*

Introduction

Several *in vitro* phototoxicity methods have been developed to assess the phototoxic potential of chemical substances. In the EU, the seventh amendment to the Cosmetics Directive (Directive 2003/15/EC) was promulgated, and the amendment has been enforced since September 11th 2004. This amendment to the Cosmetics Directive included two prohibitions: the animal testing ban of finished cosmetic products in the EU territory; and the marketing ban of cosmetic products containing ingredients which have been subject to animal testing using a method other than an alternative method after such alternative methods have been validated and adopted in the EU territory. Therefore, it is expected that the development of alternative methods with high reliability will be promoted in the future.

The Balb/c3T3 mouse fibroblast neutral red uptake phototoxicity test (3T3 NRU PT), which was developed based on the research of the mechanism of the occurrence of phototoxicity, is used to evaluate the cosmetics ingredients as an alternative method that has already been validated in the EU. This method has been incorporated into the dangerous substance directive annex of the chemicals.

Moreover, 3T3 NRU PT was slightly modified from the original method, and was accepted as an OECD in vitro phototoxicity test guideline (adoption of the OECD TG 432 2004 April 13) for chemical substances (Fig.1).

At present, this alternative to phototoxicity testing is one of the most successful pieces of alternative research when compared to other safety testing methods.

In Japan, the peer review of *in vitro* 3T3 NRU PT was executed by the scientific research group of the Japanese Ministry of Health, Labor and Welfare, and the features and the limits of this method were summarized (Ohno *et al*., 2004). As a result, it was concluded that this method was useful as a screening method using cultured cells. Furthermore the results obtained in this research were discussed at an open symposium (Ohno *et al*., 2002).

In addition, a Japanese cosmetic company proposed a battery test system (Yeast-RBC assay) (Mori *et al*., 2004 a) consisting of the combination of the yeast growth inhibition phototoxicity (YGI

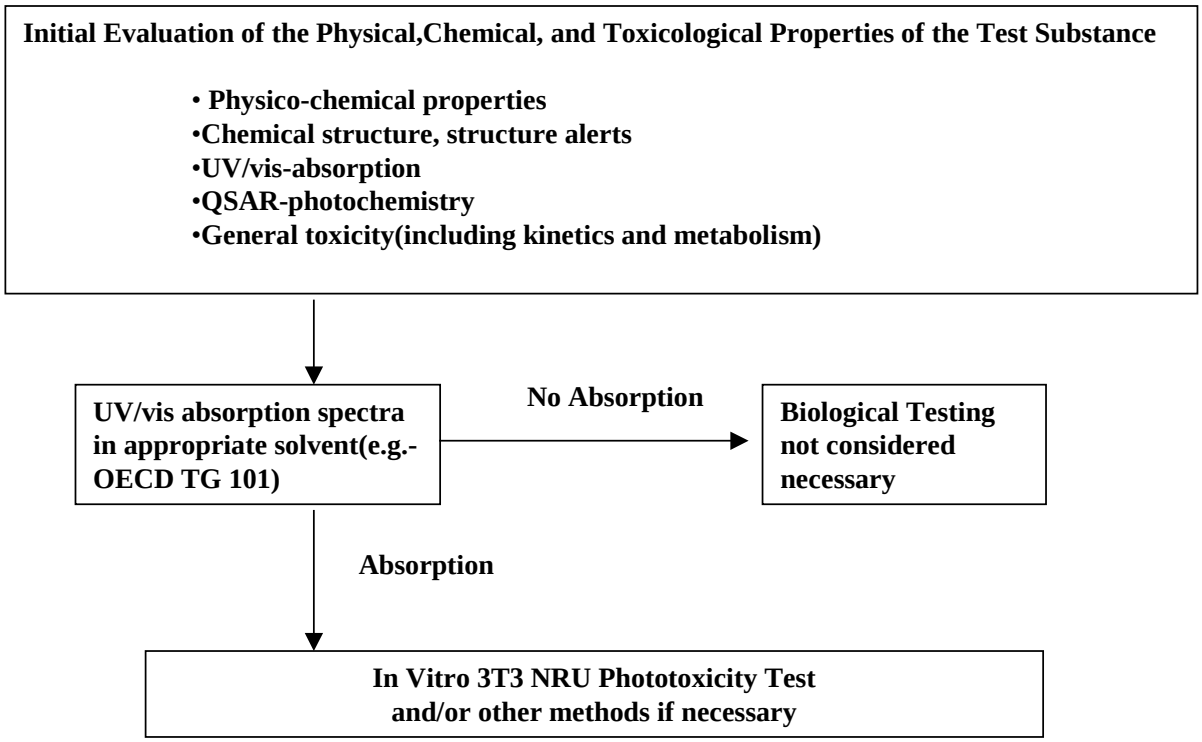

Fig.1 3T3 NRU PT in phototoxicity evaluation of chemical substance of OECD TG 432

PT) assay and the red blood cell photohemolysis (RBC PH) assay to the public advertisement of the scientific research group of the Japanese Ministry of Health, Labor and Welfare.

The review of this method was performed by an Assessment Committee of the Japanese Society for Alternatives to Animal Experiments (JSAAE), and the subsequent validation study was consigned to a Validation Committee of JSAAE.

The validation study was executed at six facilities. The interim report was presented at the science annual meeting of JSAAE (Mori *et al*., 2004 b, Yoshimura *et al*, 2004).

In this text, the approach of development of *in vitro* phototoxicity assays in Japan is described around not only the peer review of 3T3 NRU PT but also the Japanese original validation study to the Yeast- RBC assay.

Current status of development of alternatives to phototoxicity testing

Various *in vitro* phototoxicity assessments are developed based on the mechanism of the phototoxicity. Spielmann (Spielmann, 2001) describes a clear summary about the mechanism of phototoxicity and phototoxicity testing methods. The 3T3 NRU PT is approved as the *in vitro* phototoxicity alternative test method established by ECVAM in a scientific manner, and has been adopted formally as a screening method of the phototoxicity since 2000. The amendment method from the original of this method was accepted as an *in vitro* phototoxicity test guideline of the chemical substance in OECD in April 2004.

On the other hand, the pre-validation study of the photohemolysis assay using the red blood cells and the hemoglobin photo peroxidation assay was performed as the mechanism evaluation methods in the EU, and the availability of these testing methods was reported (Spielmann *et al*., 2000).

In Japan, Sugiyama et al., are reporting a lot on battery testing systems that combine the mechanism testing method to screening method like the yeast photo growth inhibition and the red blood cell photohemolysis (Sugiyama *et al*., 1994 a, 1994 b, 2002, Mori *et al*., 2004 a).

Moreover, Tanaka and Wakuri presented on the application of the 3T3 NRU PT to the photo-

toxicity evaluation of the environmental pollutants.

We evaluated the 3T3 NRU PT, the red blood cells hemolysis assay, and the hemoglobin photo peroxidation assay (Okamoto *et al*., 1999a). As a result, we confirmed that the correspondences of these results and *in vivo* data were comparatively good, respectively. The influence of the reactive oxygen species including singlet oxygen emission on the *in vitro* phototoxicity was also evaluated in order to evaluate the mechanism in these assays, and good results were obtained (Okamoto *et al*., 1999 b). In addition, we tried the application of the commercial skin models for phototoxicity evaluation of the cosmetic formula, and showed that the skin models were suitable to evaluate the phototoxicity of the water-insoluble substances or the compounds including powder materials (Okamoto *et al*., 2003).

The peer Review of 3T3 NRU PT in Japan

Outline of 3T3 NRU PT

A permanent mouse fibroblast cell line, Balb/c 3T3, clone 31 is recommended as a using cell in the OECD guidelines. The test substance solutions are added to each microtiter well, and the cells are irradiated with Sunlight（cut UVB）in the dose of 5 J/cm^2 (as measured in the UVA range). The non-irradiated microtiter plates, the cells of which have been exposed to the same test substances, are used as negative controls. After irradiation, the test substance solution is replaced with a culture medium. After incubation for 24 hours, the neutral red solution is added to each well and the neutral red uptake is determined by comparing the absorbance of test culture with that of the untreated control culture at 540nm.The IC50 (concentration causing 50% reduction in neutral red uptake, compared to the control) value is calculated from the concentration-response curve.

Phototoxicity is evaluated using the photo irritation factor (PIF) or mean photo effect (MPE).

A test substance with a PIF < 2 or an MPE < 0.1 predicts: "no phototoxicity".

A PIF >2 and < 5 or an MPE > 0.1 and < 0.15 predicts: "probable phototoxicity" and a PIF > 5 or an MPE > 0.15 predicts: "phototoxicity".

The peer review and considerations in 3T3 NRU PT

The research group of the Japanese Ministry of Health, Labor and Welfare examined the validity about the 3T3 NRU PT based on the validation research by EU/COLIPA (Spielmann *et al*., 1994 a, 1994 b, 1998 a, 1998 b).

As a result, the research group of the Japanese Ministry of Health, Labor and Welfare reported that the 3T3 NRU PT is in good correspondence with *in vivo*, and useful for screening to evaluate the presence of the phototoxicity of the experimental material (Ohno *et al*., 2004).

On the other hand, the demerit of the testing method clarified through these evaluations was as follows:

1 It is necessary to select an appropriate solvent at the adoption of this method.
2 It might be difficult to evaluate the pigmentation material and the volatile substance correctly.
3 This method is unsuitable for the material that needs metabolic activation for the phototoxicity occurrence.
4 This method is suitable to use as a screening method, because the detection of dose-dependent photoreactions is difficult.
5 The modality of the light source and the confirmation of the absorption wavelength of the test substance are indispensable to secure the reliability of the result.

Therefore, it is preferable to note these points, and to execute an enough preliminary examination with the equipment used in each facility when the 3T3 NRU PT is introduced.

Implementation of 3T3 NRU PT

Some trials were carried out to introduce 3T3 NRU PT as a screening method in our laboratory. A part of those efforts is shown as follows:

Influence of light sources and cell lines

It is important to examine the cell type and the light source used when the phototoxicity is evaluated. Though light is classified into UVB, UVA, visible light, and infrared rays, the light that plays the main role in phototoxicity is ultraviolet. Ultraviolet light (mainly UVA) is generally used as a light source in the phototoxicity testing of animals and the photo inspection of the medical practice. However, it is preferable to use a light source including visible light because the phototoxicity reaction by the visible light is reported.

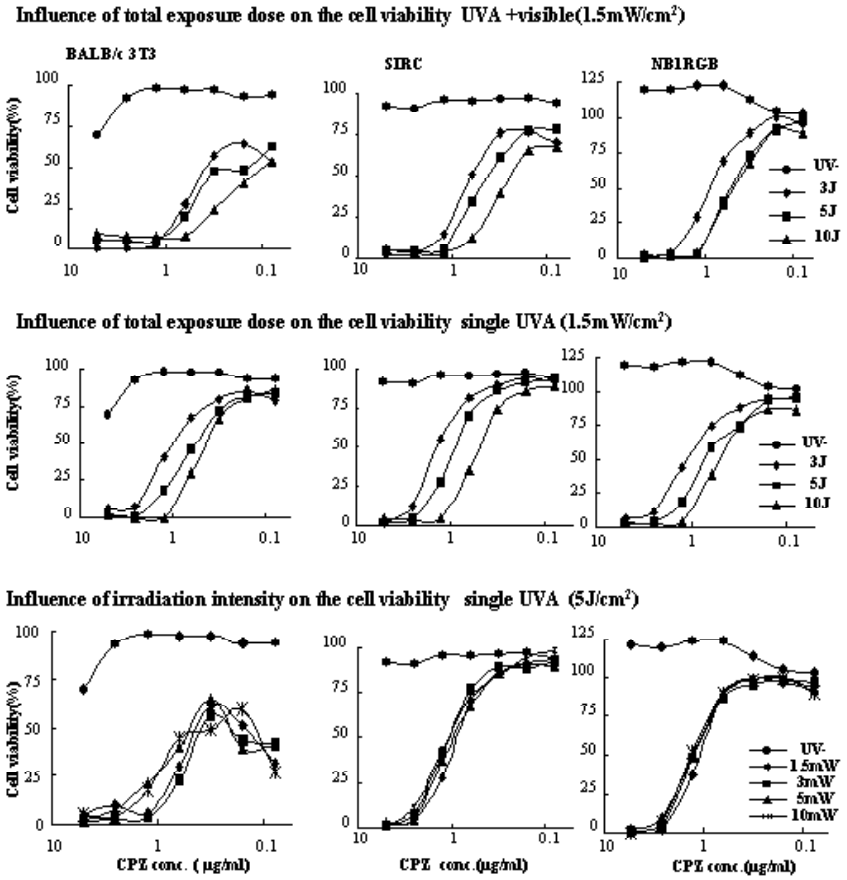

Fig.2 Influence on phototoxicity of chlorpromazine hydrochloride (CPZ) in cell lines and light exposure conditions (Imai *et al*., 2004)

On the other hand, it is necessary to understand the photosensitivity of the cell line being used. Then, the 3T3NRU PT using three kinds of cells (Balb/c 3T3: the mouse fibroblast and SIRC: the rabbit corneal cell and NB1RGB: the human fibroblast) was attempted by using chlorpromazine hydrochloride as a positive control material (Imai *et al.*, 2004). The influence of the irradiation intensity and the exposure dosage on the cytotoxicity was evaluated by using two kinds of light sources (Fig.2).

The UVA + visible light (The mercury metal halide lamp: Hönle UV Technology and SOL500 RF2) and the single UVA light (The xenon lamp: WACOM bio solar simulator WBS-85 used a cutting filter, extracting only UVA) were used as light sources. In this examination, the difference of the sensibility with the cell line was small. In the UVA + visible light, the cellular damage by the influence of the extent of heat release was severe, and it was difficult to set the irradiation intensity high. When the irradiation intensity was set to 1.5mW/cm^2 as an amount of the UVA radiation, a decrease in the cell viability according to an increase of the dose was confirmed on each cell. Moreover, the cell viability of each concentration in UVA + visible light irradiation shows a low tendency compared with those of single UVA, and it shows that the influence of UVA + visible light on each cell is a little high. The variation of the cell viability is hardly seen even if the irradiation intensity is changed when the exposure dose is fixed to 5J/cm^2 by using single UVA light.

In the single UVA light irradiation, the influence of the exposure dose was larger than the irradiation intensity in the cytotoxicity. The difference of the judgment of phototoxicity in each light source was confirmed only to the test substance with absorption in the visible light. In other test substances, no great difference was confirmed to the judgment of the phototoxicity.

As mentioned above, it is important to improve the reliability of the result by understanding the features of the modality of the test substances and the light source used when the testing method is adopted.

Application of 3T3 NRU PT to the assessment of fragrance materials

Fragrance materials are important ingredients in cosmetic products. The phototoxicity properties of such materials in cosmetics are also well known. We evaluated the *in vitro* phototoxicity of nine natural essential oils classified as phototoxic according to the International Fragrance Association (IFRA) guidelines using the 3T3 NRU PT (Okamoto *et al*., 2002). Among the negative results, six of these are obtained from the *rutaceae* family of plants and one is obtained from *umbelliferae*

Table 1 The results of the 3T3 NRU phototoxicity test and contents of psoralen derivatives in naturtal essential oils (Okamoto *et al.*, 2002)

No.	Natural essential oils	PIF value	Contents (ppm)					*in vivo* phototoxicity [a]
			Bergapten	Psoralen	Angelicine	Imperatolin	Total	
1	Angelica root oil	1.19	1.3	0.8	1.2	2.0	5.3	-
2	Bergamot oil cold pressed	2.17	33.5	n.d.	n.d.	n.d.	33.5	+
3	Bergamot oil expressed	0.94	2.3	n.d.	n.d.	n.d.	2.3	-
4	Bitter orange oil expressed	1.18	0.7	n.d.	n.d.	n.d.	0.7	-
5	Cumin oil	19.0	n.d.	n.d.	n.d.	n.d.	n.d.	+
6	Grapefruit oil expressed	1.63	1.9	n.d.	n.d.	n.d.	1.9	-
7	Lemon oil cold pressed	1.04	0.5	0.7	n.d.	n.d.	1.2	-
8	Lime oil expressed	3.38	14.4	0.8	n.d.	3.3	18.5	+
9	Marigold absolute	1544	n.d.	0.5	n.d.	3.4	3.9	+

n.d. : not detected

PIF; photoirritation factor

PIF values present the mean of two separate trials.

a; guinea pig phototoxicity test result (existing data :20% petroleum)

family of plants (Table 1). The *rutaceae* family and *umbelliferae* family of plants contain psoralen derivatives with high frequencies; their ability to cause phototoxicity is thought to be due to those psoralen derivatives (Kavli and Volden, 1984). Under IFRA guidelines, the amount of bergapten is restricted to less than 15ppm in consumer products. The bergapten content of crude bergamot is reported as approximately 3000-4000ppm (Marzulli and Maibach, 1970). The contents of four psoralen derivatives including bergapten in each essential oil were analyzed using GC-MS in this study. The psoralens used as standard substances were obtained from Extrasynthese (Genay Cedex, France) and Latoxan (Rue Leon Blum, Paris, France).

The amounts of psoralen derivatives in each natural essential oil used were at a lower level than the previously reported. The highest amount of psoralens was 33.5ppm of Bergamot oil cold pressed in this research. Two of the essential oils contained more than 15ppm of psoralens. Those were Bergamot oil cold pressed and Lime oil expressed, and their PIF values were 2.17 and 3.38, respectively. Other essential oils had amounts of less than 15ppm. To evaluate the suitability of 3T3 NRU PT to bergapten in fragrance materials is an important task, as bergapten is the one of the main causes of phototoxicity reactions in fragrance materials. The correlation between the amount of bergapten in the essential oil and the PIF value was evaluated using the addition of bergapten to Bergamot oil expressed used as the standard essential oil (Fig.3). The amount of bergapten required to result in a PIF value 5 was more than 1000ppm. The concentration of bergapten that caused the phototoxicity reaction in vivo has been reported as being more than 100ppm (Marzulli and Maibach, 1970). The PIF value at 100ppm of bergapten was

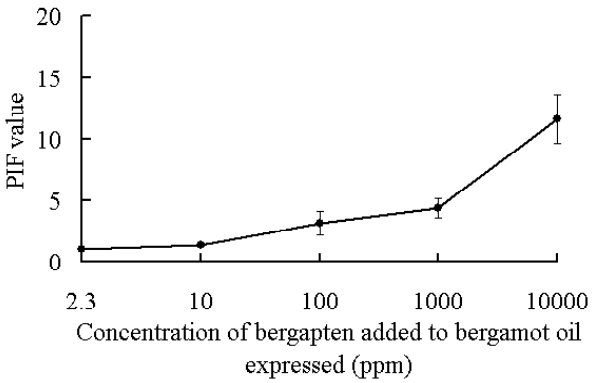

Fig.3 The correlation between the content of bergapten added to the essential oil and the PIF value. Bergapten were added to bergamot oil expressed used as a standard substance. The PIF values in each concentration of bergapten present mean with standard error of four trials. (Okamoto et al.,2002)

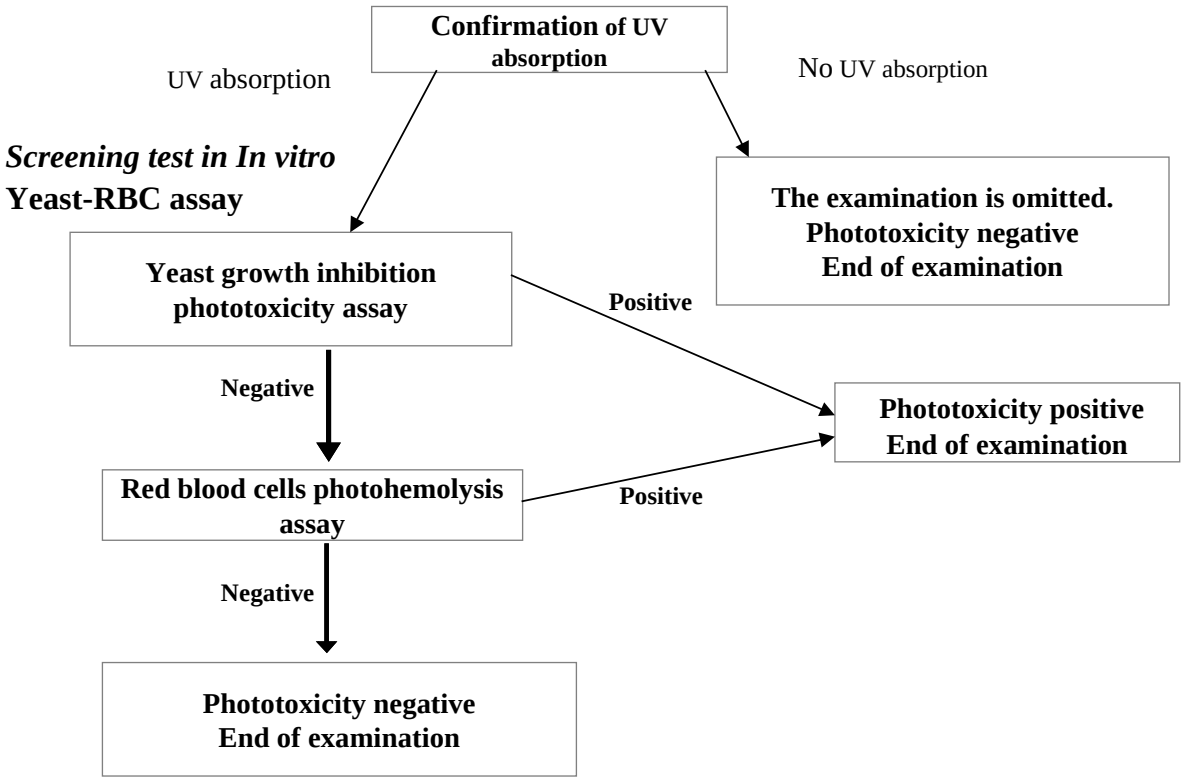

Fig. 4 Yeast-RBC assay battery system (Mori *et al*., 2004 b)

3.13. These suggest that even those with a PIF value less than 5 may cause phototoxicity *in vivo*. Therefore, in the case of the natural essential oils containing bergapten, the suitable PIF value as a cut off point was thought to be 2.

On the other hand, Marigold Absolute is of the *tagetes* family. The phototoxic ingredients of the *tagetes* family of plants are thought to be polyacethylenes and the thiophene derivatives (Chan *et al*., 1975). Alpha-terthienyl is well known as a severe phototoxicant in the *tagetes* family (Towers *et al*., 1979, Rampone *et al*., 1986). We have also confirmed the *in vitro* phototoxicity reaction of alpha-terthienyl using the 3T3 NRU PT (data not shown). Though the analysis of alpha-terthienyl content in Marigold absolute was not executed, it is guessed the *in vitro* phototoxicity of marigold absolute may be caused by alpha-terthienyl.

The evaluation of the new *in vitro* phototoxicity battery test system by the JSAAE

The primary screening of the proposed battery test system

The 3T3 NRU PT has the limit in the subject that can adjust because it is a monolayer culture system. Therefore, the development of the testing method that can solve the shortcoming of the testing method is hoped for.

Aiming to solve this problem, a Japanese cosmetic company proposed the Yeast-RBC assay consisting of the combination of the yeast growth inhibition phototoxicity (YGI PT) assay and the red blood cell photohemolysis (RBC PH) assay to the research group of the Japanese Ministry of Health, Labor and Welfare as a new phototoxicity testing method alternative. The YGI PT assays based on the evaluation of the damage to DNA or cell organelles is applicable to water-insoluble test chemicals. The RBC PH assay is based on the mechanism of destruction of the cell membrane. The assessment committee of the JSAAE accepted this proposal, and the primary screening was performed.

The following advantages were shown in the proposal battery testing system by the primary screening:

1 The testing method is comparatively easy, because the cultured cells are not used for the testing method.
2 It is possible to adjust to the low water solubility material.

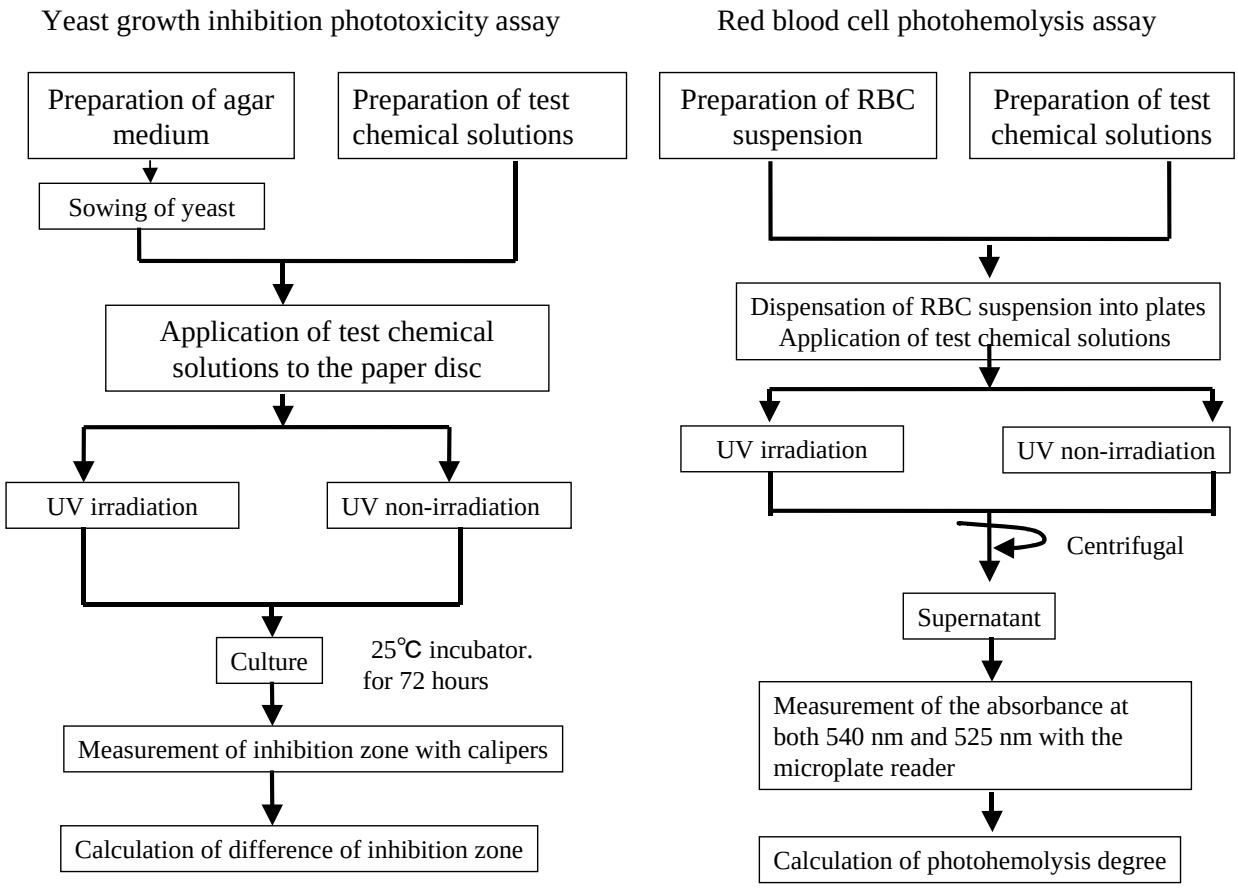

Fig. 5 The scheme of Yeast-RBC assay

3 The testing method is available for not only cosmetics and raw materials but also general chemical substances.
4 The phototoxicity based on the different toxicity mechanisms can be detected by using the combination of the two testing methods having different characteristics.

From the confirmation that the most embarrassed false negative was not detected in the submitted documents, the assessment committee judged that the Yeast-RBC assay was a useful screening method for the evaluation of the phototoxicity potential of the chemical substance.

The assessment committee also decided that the enforcement of the validation research in multiple facilities was necessary to confirm scientific validity of the testing method conclusively, and consigned the multi facilities validation to the Validation Committee of the JSAAE.

Validation researches in multiple facilities

This validation study was executed aiming to confirm the following main points: The first is to understand the level of the inter-laboratory reliability in the multi facilities quantitatively; The second is to examine the appropriateness of the instruction of standard operating procedure (SOP) of the proposed battery system, especially, adequacy of positive and negative judgment criteria.

Test protocols

The revision of the protocol was performed by the proposer according to the addition of the improvement matter pointed out by the primary screening before the validation study (Fig.4 and 5).

The reliability of the inter-laboratories

In the validation study, the reproducibility of the inter-laboratories was relatively good. The improvement of the false positive by the battery evaluation was confirmed at six experiments of 36

Table 2 Multi facilities validation study

Validation committee:
H. Itagaki, Y. Ohno, T. Omori, Y. Okamoto, R. Kawabata, H. Kojima, N. Tanaka, T. Doi, Y. Fujita, M.Hoya, M. Mori, S. Wakuri and I. Yoshimura (chairman)

Data analysis: T. Omori and I. Yoshimura (chairman)

Participants
- Shiseido Co. Ltd Ltd.
- Kosé Corporation Ltd.
- Food and Drug Safety Center
- Toyo Beauty Co. Ltd.,
- Nippon Menard Cosmetic Co. Ltd.,
- Maruho Co. Ltd.,

Test chemicals
- nine materials

*The sample is encoded and distributed by the Validation committee.

Light source
- Solar simulation light;SOL500 (Dr. Hönle GmbH, Martinsried, Germany)

*The examination was executed esteeming the GLP.
* A technological transfer was carried out.

Results
Inter-laboratory variation
–The range of differences of the judgment in each laboratory had been included within a positive and a false positive, or a false positive and a negative range.
Validity of Yeast-RBC assay
–Sensibility: 100%
–Specificity : 47%
–Equivalence: 70%.

Summary
–Yeast-RBC assay is useful to predict the phototoxic potential of chemicals from the viewpoint that there is no false negative, when equivocal data were treated as positive.
–The improvement of the false negative by the battery evaluation was confirmed at six experiments of 36 experiments as a whole, when equivocal data were treated as positive.

experiments as a whole. Therefore, it was shown that the Yeast-RBC assay was a possible alternative screening method to predict the phototoxicity substances (Table 2).

The concerning point, which should be clarified when putting this system to the practical use as a screening method, is being discussed including feedback to the proposer now.

Conclusion

The development of the alternative to phototoxicity testing is advanced compared with other safety testing alternatives.

The seventh amendment of the EU has been enforced, and the acceptance of these alternative test methods to screening will advance in Japan in the future.

It is important to understand the feature of the experimental material clearly, and to build an appropriate alternative into the safety assessment to obtain a useful result when each facility introduces these alternative test methods.

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