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Ocular Toxicity Assessment of Bisphenol A and Its Derivatives By The Hen's Egg Test - Chorioallantoic Membrane (HET-CAM) assay

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Abstract

Bisphenol A (BPA) and its derivatives, Bisphenol F (BPF), Bisphenol S (BPS) are classified as endocrine disruptor chemicals that can affect with the human in different ways. In raising concerns about the adverse health effects of these chemicals, no ocular irritation data is available in the literature. It was aimed to reveal the comparative irritation potential of BPA and its analogues for the first time with HET-CAM test, which is an alternative method to the *in vivo* Draize test. Irritancy or alterations of BPA, BPF and BPS were detected by observing adverse changes such as hemorrhage, coagulation, or lysis of blood vessels on the CAM surface. Test results indicate that BPA caused moderate irritation characterized by lysis with an invisible vein (IS 6.9 ± 0.05), whereas BPS had a strong irritation impact appearing as hemorrhage and coagulation (IS 11.8 ± 0.02). There was no irritancy after BPF exposure on the CAM surface. As a conclusion, it was supported that BPA and BPS serious toxic chemicals for human health when considering exposure that may be higher for workers. At the same time, this study is significant since it offers an alternative approach to animal studies for evaluating the irritation potential of bisphenols, particularly in the context of industrial exposures.

Keywords

Bisphenol,
Toxicity,
Irritation,
in ovo,
HET-CAM

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Bisfenol A ve Türevlerinin Oküler Toksisitesinin Tavuk Yumurtası Testi - Koryoallantoik Membran (HET-CAM) Testi ile Değerlendirilmesi

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Öz

Bisphenol A (BPA) ve türevleri olan Bisphenol F (BPF), Bisphenol S (BPS), insanları farklı şekillerde etkileyebilen endokrin bozucu kimyasallar olarak sınıflandırılmaktadır. Bu kimyasalların olumsuz sağlık etkileri hakkında endişeler dile getirilirken, literatürde göz tahrişine dair veri bulunmamaktadır. *In vivo* Draize testine alternatif bir yöntem olan HET-CAM testi ile BPA ve analoglarının karşılaştırmalı tahriş potansiyelini ilk kez ortaya koymak amaçlanmıştır. BPA, BPF ve BPS'nin tahrişi veya değişiklikleri, CAM yüzeyinde kan damarlarında kanama, pıhtılaşma veya lizis gibi olumsuz değişiklikler gözlemlenerek tespit edildi. Test sonuçları, BPA'nın görünmez bir damarla lizis ile karakterize orta düzeyde tahrişe neden olduğunu (IS 6.9 ± 0.05), BPS'nin ise kanama ve pıhtılaşma şeklinde görünen güçlü bir tahriş etkisine sahip olduğunu göstermektedir (IS 11.8 ± 0.02). BPF maruziyetinden sonra CAM yüzeyinde herhangi bir tahriş oluşmadı. Sonuç olarak, işçiler için daha yüksek olabilecek maruziyet dikkate alındığında, BPA ve BPS'nin insan sağlığı için ciddi toksik kimyasallar olduğu desteklenmiştir. Aynı zamanda, bu çalışma, özellikle endüstriyel maruziyetler bağlamında, bisfenollerin tahriş potansiyelini değerlendirmek için hayvan çalışmalarına alternatif bir yaklaşım sunduğu için önemlidir.

Anahtar kelimeler

Bisphenol,
Toksisite,
İrritasyon,
in ovo,
HET-CAM

1. INTRODUCTION

The rising influx of chemicals into the market and environment demands the surveillance of environmental materials, specimen acquisition, and the creation of swift and accurate methodologies for toxicity evaluation. Over the past three decades, various alternative methodologies have been created and substantiated, encompassing in vitro, ex vivo, and in silico techniques, to evaluate the toxicity of cosmetics, pesticides, and plastics, particularly concerning ocular mucosa [1]. Due to the tissue in the eyes being so sensitive, the safety of ophthalmic products must be evaluated alongside their efficacy. The Draize test in rabbits has long been the standard paradigm to determine the risk of ocular irritation [2]. However, it is now forbidden to use animals to test the safety of cosmetics for obvious ethical reasons. Draize test can inflict excruciating agony and suffering for a few days. The Hen's Egg Test- Chorioallantoic Membrane (HET-CAM) is an alternative test to Draize, a quick, sensitive, and reasonably priced toxicity test that can provide details on the effects of chemicals on the developing embryo, teratogenicity, systemic and immunopathological consequences, metabolic pathways, and, recently, the potencies of chemicals that irritate mucosal membranes [3] [4]. The HET-CAM test is an alternative assay designed by EURL-ECVAM (European Union Reference Laboratory for Alternatives to Animal Testing) to assess the level of irritant impact. It allows to observe the potential effect of compounds through changes as vascular damage on the chorioallantoic membrane of the egg after exposure to the test sample.

Worldwide demand for food containers, metal cans, dental sealant composites, medical equipment, and other items made of polycarbonate plastics and epoxy resin is encouraging the use of bisphenol A and its derivatives as an organic synthetic plasticizer. BPA mimics estrogen to disrupt metabolic processes and alter the body's dynamic equilibrium, which is why it is classified as an endocrine disruptor [5]. Nowadays, BPF and BPS have replaced BPA in products including water pipe coatings, thermal paper for receipts, and inside coatings of food, pharmaceutical, and cosmetic containers. Even, in 2018, The European Chemicals Agency (ECHA) conducted a market survey to ascertain the volume of BPS utilized as a developer in thermal paper produced in the European Union (EU), following the substitution of BPA with BPS by EU paper manufacturers [6]. However, there is still less information about potential adverse health outcomes after occupational exposure.

Given the extensive utilization of bisphenols across various industrial applications, occupational exposure and the associated health risks to workers are significant concerns [6]. Moreover, the exposure levels to bisphenols may be elevated for workers who manipulate it compared to the general population. In the study of [6] addressed BPA occupational exposure, it was mentioned that BPA may accumulate in the skin, offering exposed individuals a continuous source for absorption. However, in the literature, some researchers demonstrated that administration of BPA and antigen via eye drops enhanced antigen uptake by antigen-presenting cells (APCs) in the tear duct-associated lymphoid tissue (TALT) [7]. When absorbed from the skin by contact, bisphenols stay in the body longer than ingested through food or drink [8]. The precise mechanism by which bisphenol, a prevalent endocrine disruptor, compromises immune cells remains unidentified. Due to considering probable exposure pathways and the lack of comparative research on the ocular irritancy of these chemicals, a detailed investigation of their comparative irritation effects is necessary. In this study, the possible ocular irritation and alteration effects of BPA and its derivatives BPF and BPS were determined for the first time using an alternative method to in vivo studies by the HET-CAM test.

2. MATERIAL AND METHOD

2.1 Materials

Bisphenol A (BPA), Bisphenol F (BPF) and Bisphenol S (BPS) were obtained from Aldrich (St. Louis, MO, USA, Alfa Aesar, Thermo Fisher GmbH, Kandel, Germany and Acros Organics). All other chemicals were obtained from Sigma-Aldrich (USA).

2.2. Hen's Egg Test on Chorioallantoic Membrane (HET-CAM) Test

The test was conducted in line with ICCVAM using Ross308 fertilized chicken eggs (50-60g) with three independent duplicates, and the eggs were incubated at 37±0.5°C and 70% humidity for 7 days [4]. On the 7th day, a window (2x2cm) was opened at the equator of the eggs and 300 µL of each chemical was applied directly onto the CAM surface and maintained contact for 0.5, 2 and 5 minutes. 0.9% NaCl solution was used as a negative control and 0.1N NaOH was used as a positive control. Following the exposure of each sample, the membrane was assessed for vascular damage, and the duration was recorded. The irritancy score was classified according to Table 1. The possible irritation degree score (IS) was calculated as follows;

$$IS = [(301 - tH) \times 5]/ 300 + (301 - tL) \times 7]/ 300 + (301 - tC) \times 9]/ 300$$

where tH, tL and tC are the time required for hemorrhage, lysis and coagulation to occur, respectively (in seconds). Depending on their IS values, formulations are classified as non-irritating (IS < 1), slightly irritating (1 ≤ IS < 5), moderately irritating (5 ≤ IS < 10), or severely irritating (IS > 10).

Table 1. Classification of irritancy score in the HET-CAM test

Cumulative Score	Scheme
0-0.9	Non-irritant
1-4.9	Slight irritant
5-8.9	Moderate irritant
9-21	Strong irritant

2.3. Statistical Analysis

Each analysis was repeated in three replicates. Statistical difference was analysed as P <0.05 significance level with one-way ANOVA followed by Duncan's multiple comparison test with using Graphpad Prism 8.0.

3. RESULTS AND DISCUSSION

The HET-CAM assay is highly appropriate for forecasting conjunctival damage since it assesses the acute effects of substances on the chorioallantoic membrane's proteins and tiny blood vessels [9]. The allantoic wall and chorion fuse to form the highly vascular embryonic membrane known as the CAM. The conventional Draize test permits the identification of irritative reactions analogous to those observed in the eye. Three reactions are observed in the HET-CAM: coagulation, lysis, bleeding on the chorioallantoic membrane on the seventh day of embryonic development, during which pain perception and nerve tissue are still maturing [10]. The HET-CAM test can assess chemical toxicity by analyzing conjunctiva damage, which functions as a vascular mucosal tissue in the human eye [11]. Toxicology testing has historically relied more on animal testing as it has grown in popularity. A variety of in vitro techniques utilizing different endpoints have been established to elucidate the intricate physiological reactions of the skin to hazardous agents [12]. These responses focus on cutaneous assessment, which has been examined through various in vivo assays, including the skin penetration test, Draize test, sensitization test in guinea pigs. To reduce dependence on live animal testing, there has been a general increase in funding for ex vivo and in vitro alternative procedures. It also created a political environment in which alternative processes were included into federal and government legislation [1]. The quantity of laboratory animals utilized for biological testing might be decreased by excluding novel compounds shown to be irritants from in vivo testing. This assay may be especially useful for assessing drugs that are intended to be delivered through the mucosa. Because products applied to mucosal surfaces shouldn't irritate them, this is a crucial consideration [13]. A study investigated the correlation between in vitro models, including the HET-CAM assay, and clinical data, and the findings showed that there is a significant positive correlation between the HET-CAM test and the human patch test results for anionic and amphoteric surfactants [11]. The present study assessed Bisphenol A and its derivatives BPS and BPF potential to cause ocular irritation in comparison by using the HET-CAM test for the first time. The findings were categorized as presented in Table 1 after scoring, and the results were summarized in Table 2.

Table 2. Irritation score and classification of test samples in the HET-CAM test

Test Sample	IS*	Classification
NC (0.9% NaCl)	0	Non-irritant
BPA	6.9 ± 0.05	Moderate irritant
BPF	0	Non- irritant
BPS	11.8 ± 0.02	Strong irritant
PC (0.1N NaOH)	13.7± 0.03	Strong irritant

* IS, irritation score; 0 ≤ IS ≤0.9, Nonirritant; 1 ≤ IS ≤4.9, Slight irritant; 5 ≤ IS ≤8.9, Moderate irritant; 9 ≤ IS ≤21, Strong irritant.

According to the test results, when the BPA, BPF, and BPS-treated groups were compared, BPS demonstrated a stronger irritation effect than BPA and BPF, particularly as the exposure time increased. While BPA induced a moderate irritation effect characterized by lysis and invisible veins after the 5th minute, BPS caused a marked irritation response, evident by pronounced hemorrhage and coagulation on the CAM surface at the same time point. This suggests that BPS may possess a higher potential for tissue damage or vascular disruption under similar experimental conditions, highlighting the need for careful toxicological evaluation of BPS as a substitute for BPA (Figure 1).

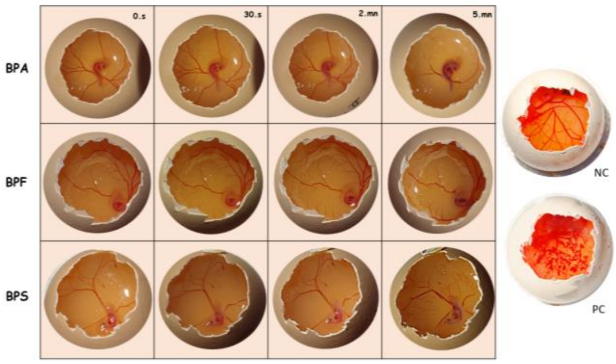

Figure 1. Effects of the Bisphenol A (BPA), Bisphenol F (BPF), Bisphenol S (BPS) and controls on the CAM surface. Legend: T0.s–time 0 minutes; T 30.s–time 0.5 min; T2.min–time 2.0 min; T5.min–time 5.0 min. NC-negative control (0.9% NaCl), PC-Positive control (0.1N NaOH).

This distinction corresponds with recent evidence suggesting that, although presented as safer substitutes for BPA, BPS and BPF still demonstrate considerable biological impacts [14]. In the literature, BPS has demonstrated estrogenic, reproductive, and developmental toxicity in various in vitro and in vivo models. Moreover, comparative toxicity studies—such as those assessing cytotoxicity via in vitro models—have found that while BPS and BPF may present lower acute cytotoxicity than BPA, they still warrant caution [15]. Although there is no study in the literature showing the comparable irritation effects of bisphenol A and its analogues, this study has been demonstrated for the first time with the HET-CAM test. Given its structural similarity to BPA, numerous researches have investigated the potential of BPF and BPS as an endocrine disruptor [16] and may exert direct influences on dermal health [17]. In our previous study on BPA and its analogues effects in breast cells, we demonstrated that BPF and BPS had greater toxic effects than BPA on human breast cells [18].

BPA can migrate from coatings and materials into food and beverages by hydrolysis or diffusion of residual BPA on polycarbonate surfaces [19]. While earlier research mostly examined human exposure to BPF by direct oral intake or inhalation, Liu and Martin (2017) discovered that bisphenol analogs have a longer duration of presence in the body when absorbed from the skin contact, on the contrary to being consumed through food or drinks [8]. Eladak et al. (2015) shown that BPF and BPS serve as alternatives to BPA by influencing cell proliferation, apoptosis, and hormonal activity [20].

The Classification, Labelling, and Packaging (CLP) Regulation identifies BPA as possibly reproductive toxic, perhaps irritating to the skin, and potentially harmful to the eyes [19]. In the United States, Bisphenol F (BPF) was identified as the second most common chemical after BPA in diverse foodstuffs, with an average content of 0.93 ng/g wet weight [21]. Most of studies reveal the presence of BPA and its analogs BPF, BPS, and BPB in canned foods, which demonstrate the highest levels of both individual and aggregate bisphenols [21]. Especially, BPS is utilized extensively in consumer applications that serves as an intermediary in the synthesis of epoxy resins and polycarbonate polymers, and is used in numerous industrial items, such as cleaning agents [22]. In the study of (Kitagawa et al., 2023), the toxicity of BPF was investigated with acute and subacute exposure to mice, and the results were shown hypersensitivity symptoms and persistent acute inflammation similar to human psoriasis [23]. In the comprehensive study, an extensive evaluation of 27 BPA analogues frequently utilized in industrial applications demonstrated that merely three compounds—BPE, BPF, and 2,4-BPS—displayed lesser sensitizing effects than BPA [24].

The substitution of BPA with structurally analogous compounds such as BPF, and BPS has received considerable traction due to public health concern surrounding BPA's endocrine-disrupting properties. When the studies were examined, it was concluded that most of the focus was on BPA, that there was a lack of studies on the irritation effects of BPF and BPS, and that guidance documents should be prepared for their safe use. Nonetheless, a consensus has yet to be established among researchers, regulatory experts, and risk managers about the health hazards posed by BPA, BPS and BPF at current exposure levels. It is clear that this study will make a significant contribution to the literature, especially since there is a lack of studies on the irritation effect of BPS with HET-CAM.

4. CONCLUSION

Utilizing the HET-CAM assay for risk assessment, bisphenols were categorized as irritating chemicals, and this classification was predicated on their undesirable reactions within the vascularization of the chorioallantoic membrane. It is obvious that there was a lack of studies in the literature, especially on BPS and BPF irritation effects, and it was concluded that guidance documents should be prepared for the safe use of bisphenol analogues in this regard. Concerning methodological design, the outcome of this study is particularly important as it highlights the potential of alternative methods, such as HET-CAM test to replace use of experimental animals, especially in the context of assessing occupational exposure to chemicals such as bisphenols.

Declaration statements

Conflict of Interest
The author(s) declare that no conflict of interest.

Ethical Statement
Not applicable.

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