

Research Article

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Investigation of the Biocompatibility of Surgical Masks

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Abstract: According to the ISO10993-1 standard medical devices should be evaluated before marketing. Although there are studies that monitor the toxicity of several marketed medical devices, none of them describe the toxicity of masks that are widely used to avoid occupational exposure to biological hazard or toxic chemicals. The aim of this study was to evaluate the biocompatibility of eight purchased surgical masks of different brands, investigating their cytotoxicity and inflammation inducing capacity. Cytotoxicity was assessed via the MTT cell viability assay and inflammation was monitored by measuring nitrite, kynurenine and tryptophan levels. A preliminary study revealed that four samples were capable of killing L929 cells. Therefore the materials composing these masks were also evaluated separately. While the exposure to non-woven materials did not involve any changes in cell survival, exposing cells to elastic and sponge materials led to death in significant levels. Also, significant increases in nitrite levels with a decrease in tryptophan and kynurenine levels were obtained with cells treated with these materials, suggesting an inflammatory response that could be related to the observed cytotoxicity. Our studies revealed that the half of the randomly collected masks did not suit the biocompatibility criteria established by the ISO10993-1 standard, which is a quite unexpected result.

Keywords: Biocompatibility; cytotoxicity; inflammation; ISO 10993; surgical masks; tryptophan.

Introduction

The U.S. Food and Drug Administration defines a medical device as an instrument, apparatus, implement, machine, contrivance, implant, *in vitro* reagent or other similar or related article which is intended for use in the diagnosis of diseases or other conditions, or in the cure, mitigation, treatment or prevention of a disease [1].

There are many regulations that have been settled by different international agencies such as the European Commission, the Center for Devices and Radiologic Health of the U.S. FDA, the Pharmaceutical and Medical Device Agency of Japan or the Turkish Medicines and Medical Devices Agency to ensure the safe use of these devices as they demand an assessment of their biocompatibility according to the International Standard ISO 10993 [2, 3].

The ISO 10993 guide has set a series of tests such as cytotoxicity, sensitization, hemocompatibility, genotoxicity or carcinogenicity tests that should be performed on the final finished form of the device for its approval. These tests have been categorized according to the nature of the device, the nature of its contact with the body (intact skin contact, mucosal membrane, tissue, bone, etc...) and the duration of its contact [4].

There are many studies that monitor the toxicity of marketed medical devices [5, 6] but the biocompatibility of marketed surgical masks has not been evaluated yet in the literature. Surgical masks are widely used disposable devices that are most commonly manufactured with non-woven material [7]. Since these items can be in contact with skin for hours during surgical interventions, in this study we aimed to evaluate the biocompatibility of eight marketed masks of different brands by investigating their cytotoxicity and inflammation inducing capacity.

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Mask type	Non-woven with elastic ear loops	Non-woven with tying strips	Non-woven with elastic strap
Picture			
Sample n°	1, 2, 3, 6, 8	4, 5	7

Figure 1: Materials of the masks (non-woven masks with elastic ear loops, non-woven mask with tying strips and non-woven mask with an elastic strap).

Materials and Methods

Sample Collection

Masks of eight different brands were collected from various health organizations, numbered and classified according to their materials into three groups: non-woven masks with elastic ear loops, non-woven mask with tying strips and non-woven mask with an elastic strap (Figure 1).

Sample Preparation

Samples were extracted in four different concentrations (12.5%, 25%, 50% and 100 %), according to ISO 10993-12 [8]. Briefly, materials were extracted with an extraction ratio of 6cm²/mL in cell culture medium for 24 hours at 37°C for materials with a thickness smaller than 0.5mm. Thicker materials (>0.5mm) were extracted with a ratio of 3cm²/mL as mentioned in the guideline. The 12.5%, 25%, 50% concentrations were obtained with serial dilution from the 100% extract using cell culture medium.

Cytotoxicity test

The L929 cells were cultured in DMEM (Gibco, England), supplemented with 10% FBS (Gibco, USA) and 1% streptomycin and penicillin (Gibco, USA) at 37°C in 5% CO₂.

Qualitative morphological grading of cytotoxicity of extracts was examined according to the ISO 10993-5 standard [9]. The achievement of a reactivity grade greater than “mild” which means that “not more than 50 % of the cells are round, devoid of intracytoplasmic granules, no extensive cell lysis; not more than 50 % growth inhibition observable”, was considered a cytotoxic effect.

As a quantitative assay, cytotoxicity was assessed via the MTT cell viability assay test protocol which is based on the measurement of the viability of cells via metabolic activity [9] L929 cells were seeded into 96-well plates and maintained in culture for 24 h to form a semi-confluent monolayer. Then, they were exposed to the four different concentrations of test sample extract. After 24 h exposure, the formazan formation was determined for each treatment concentration and compared to that of control cultures. A decrease in the number of living cells resulted in a decrease in the metabolic activity of the cell and this was monitored by the optical density at 570 nm. The cell viability ratio was determined to be the ratio between the absorbance of the tested well and the absorbance of the control well by using following equation:

$$\text{Cell viability (\%)} = 100 \times \text{OD}_{570e} / \text{OD}_{570b}$$

OD_{570e} is the mean value of the measured optical density of the 100 % extract;

OD_{570b} is the mean value of the measured optical density of the blanks.

The 100 % extract of the test sample with at least the same or a higher viability than the 70 % of the blank was evaluated as having no cytotoxic potential.

The Effects of Exposure Time

The effects of exposure time on cytotoxicity and induction of pro-inflammatory response were evaluated after 1, 2 and 4 hours of exposure to the 100% sample extract. L929 cells were seeded into 48-well plates at 2x10⁴cells/well and maintained in culture for 24 h to form a semi-confluent monolayer. After exposure for 1, 2 or 4 hours (37°C, 5% CO₂), medium was collected for the analysis of releasing pro-inflammatory markers, nitrite and kynurenine (Kyn)

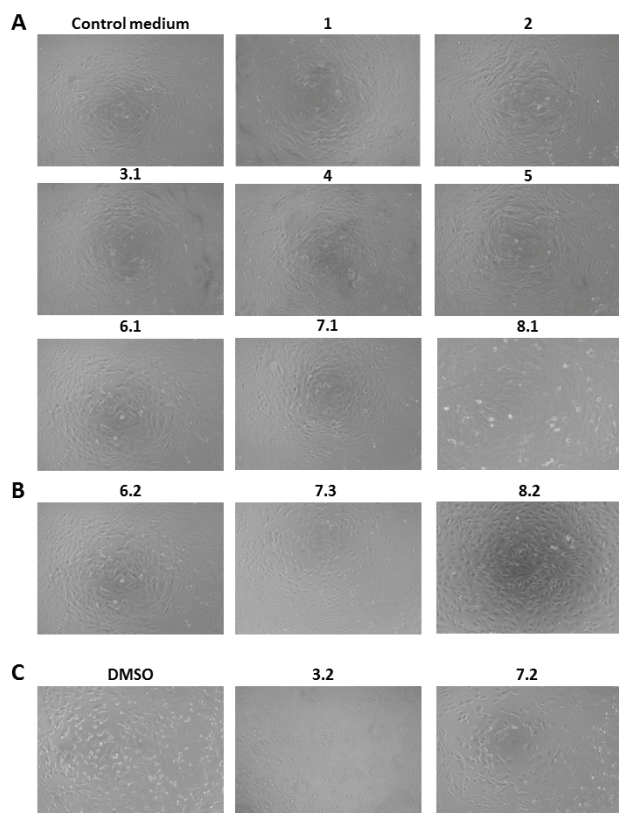


Figure 2: Morphological change of cells when exposed to different mask extracts (10x). (A) Exposure to mask sample extracts obtained from non-woven material did not show any cytotoxicity (1, 2, 3.1, 4, 5, 6.1, 7.1, 8.1); (B) Moderate cell death was also observed with strap and sponge extracts of samples 6, 7 and 8; (C) L929 cells treated with elastic straps of sample 3 and 7 were severely killed as did cells treated with 20% DMSO.

to tryptophan (Trp) ratio and exposure was stopped by rinsing with DPBS. Fresh medium was added and incubated for another 23, 22 or 20 hours respectively and the MTT assay was then performed.

Determination of Nitrite Levels:

Inflammation was monitored by measuring the stable nitric oxide (NO) metabolite levels, nitrite levels, in supernatants of cells exposed to 100% sample extracts with some modification [10]. The culture supernatant (50 μ L) was mixed with Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma-Aldrich, USA) in 5% phosphoric acid (Mettler, Switzerland)] and incubated at room temperature for 10 minutes. The absorbance of the mixture was determined at 570 nm using a microplate reader (Multiskan Ascent,

Finland). The amount of nitrite in the test samples was calculated using a sodium nitrite (Fluka Chemika, Germany) standard curve.

Determination of Kynurenine and Tryptophan Levels

Trp and Kyn concentrations in supernatant were analyzed by high performance liquid chromatography (SHIMADZU Prominence LC-20A HPLC, Kyoto, Japan) [11]. Trp was detected with a fluorescence detector (excitation 286 nm, emission 366 nm) and Kyn was detected with a UV detector at 360 nm. The Trp and Kyn concentrations were both expressed in μ mol/L. The ratio of Kyn/Trp was calculated to estimate the activity of indoleamine 2,3-dioxygenase (IDO) as the degree of Trp degradation and expressed as μ mol Kyn per mmol Trp.

Statistical Analysis

Results were expressed as mean \pm standard deviation (Mean \pm SD). The comparison between two independent groups was done using the Paired Samples T-Test. p-value $<$ 0.05 was considered statistically significant. For each compound, three independent experiments were performed with each test compound and with two parallels.

Results

Cytotoxicity

A preliminary study revealed that four of the collected samples (1, 2, 4 and 5) were not cytotoxic while extracts obtained from the remaining four samples were capable of killing L929 cells (3, 6, 7 and 8). To investigate the origin of this toxicity in more detail, the material composition of these masks was also evaluated separately. The results obtained after exposing the extracts (100%) to the cells are shown in Figure 2. Pictures clearly indicate that the elastic ear loops of samples 3, 6 and 8 (numbered as 3.2, 6.2 and 8.2) and the strap and sponge part of sample 7 (numbered as 7.2 and 7.3) present cytotoxicity.

The cytotoxicity of the samples was also determined quantitatively using an MTT assay and results are summarized in Table 1. 20% DMSO was chosen as the

Table 1: Relative cell viability (% of medium control) changing over time of exposure.

Cell viability (%) with different exposure times				
Sample No	1 hour	2 hours	4 hours	24 hours
Medium control (NC)	100 ± 6	100 ± 8	100 ± 6	100 ± 5
%20 DMSO (PC)	39 ± 5	35 ± 3	33 ± 3	11 ± 2
1	103 ± 5	109 ± 6	102 ± 7	84 ± 4
2	101 ± 5	103 ± 10	94 ± 4	88 ± 5
3.1	105 ± 5	111 ± 8	100 ± 1	89 ± 3
3.2	38 ± 4	34 ± 10	30 ± 3	23 ± 2
4	102 ± 4	113 ± 10	102 ± 5	91 ± 3
5	102 ± 5	109 ± 8	95 ± 5	94 ± 5
6.1	100 ± 5	114 ± 4	106 ± 2	87 ± 4
6.2	101 ± 4	110 ± 4	93 ± 11	66 ± 2
7.1	98 ± 2	114 ± 2	110 ± 3	95 ± 3
7.2	36 ± 6	40 ± 1	34 ± 1	26 ± 3
7.3	107 ± 7	104 ± 5	106 ± 0,5	61 ± 4
8.1	106 ± 6	114 ± 4	110 ± 3	103 ± 7
8.2	110 ± 4	108 ± 4	111 ± 3	67 ± 2

Results shown are as mean values ±SD of three independent experiments run in duplicates. Values in bold indicate significant decrease when compared to medium control; p<0.05.

Table 2: Inflammation markers changing over exposure time.

Sample No	Nitrite levels (µM)			Trp (µmol/L)	Kyn(µmol/L)	Kyn/Trp
	1 hour	2 hours	4 hours	4 hours	4 hours	4 hours
Medium control (NC)	23.7±2.5	26.0±1.2	24.4±1.0	68.9±7.6	0.91±0.14	13.2±1.3
%20 DMSO (PC)	21.3±0.3	27.8±0.8	23.8±0.3	58.7±6.3	0.59±0.13	10.5±2.1
1	21.9±0.5	26.5±1.2	25.9±1.7	69.7±5.8	0.95±0.06	13.7±0.6
2	21.7±0/5	23.6±0.6	23.6±0.3	71.1±0.8	1.12±0.06	15.7±0.5
3.1	21.4±0.3	24.4±0.8	24.4±0.8	71.2±6.5	1.16±0.16	14.3±0.6
3.2	44.9±1.6	50.6±1.9	48.3±1.7	61.4±2.3	0.60±0.07	9.7±1.5
4	21.0±0.5	23.3±0.3	23.3±0.3	63.5±6.1	0.84±0.07	13.2±0.2
5	22.0±1.2	25.4±1.3	24.6±1.1	71.5±5.3	1.04±0.03	14.6±0.8
6.1	22.5±2.3	24.3±0.3	24.4±1.7	74.6±8.4	1.0±0.12	13.5±0.1
6.2	20.8±0.3	24.1±0.3	23.5±0.8	73.2±1.5	1.05±0.10	14.4±1.1
7.1	23.2±0.1	26.3±0.5	26.8±0.5	72.8±1.2	0.89±0.10	12.2±2.1
7.2	34.0±0.7	36.7±1.5	36.8±0.8	65.1±1.9	0.68±0.01	10.5±0.3
7.3	66.1±0.3	73.5±0.8	73.5±1.7	61.1±1.7	0.47±0.04	7.7±0.8
8.1	23.4±1.1	25.2±0.7	26.0±0.7	69.9±6.4	0.87±0.04	12.5±1.1
8.2	21.4±1.6	27.8±1.0	27.4±0.5	67.7±1.6	0.96±0.06	14.1±1.4

Result are given as mean ± SD. Values in bold indicate significant decrease when compared to medium control; p<0.05.

Trp:Tryptophan; Kyn:Kynurenine; Kyn/Trp:Kynurenine to tryptophan ratio; DMSO:Dimethyl sulfoxide

positive control and gave a reproducible cytotoxic response as a cell viability of only 11 ± 2% was observed after 24 hours of exposure. Cells plated with the suitable growth medium were healthy and the cell viability remained at 100± 5%. Exposure of the cells to non-woven material, extracts resulting from the mask itself or the tying straps, did not lead to any cytotoxicity, since the cell viability remained around 100%. To quantify the cytotoxicity of the extracts obtained from the elastic and spongy materials composing the evaluated surgical masks, the cell viability was compared to the negative control and were found to be moderately or strongly cytotoxic since the ISO standard describes materials as being toxic as soon as their extract lead to more than 30% cell death. Therefore samples 6.2, 7.3 and 8.2 can be considered as moderately cytotoxic while samples 3.2 and 7.2 show significant cytotoxic effects.

The effect of exposure time on cytotoxicity was also evaluated by additional studies (Table 2). Extracts from three elastic straps (6.2, 7.2 and 8.2) did not present any cytotoxic response in the first four hours however, cell viability decreased below 70% after 24 hours exposure. We observed that for all cytotoxic materials cytotoxicity increases with the exposure time.

Inflammatory activity

The possible inflammatory activity of the masks was assessed by measuring the NO levels of the supernatant

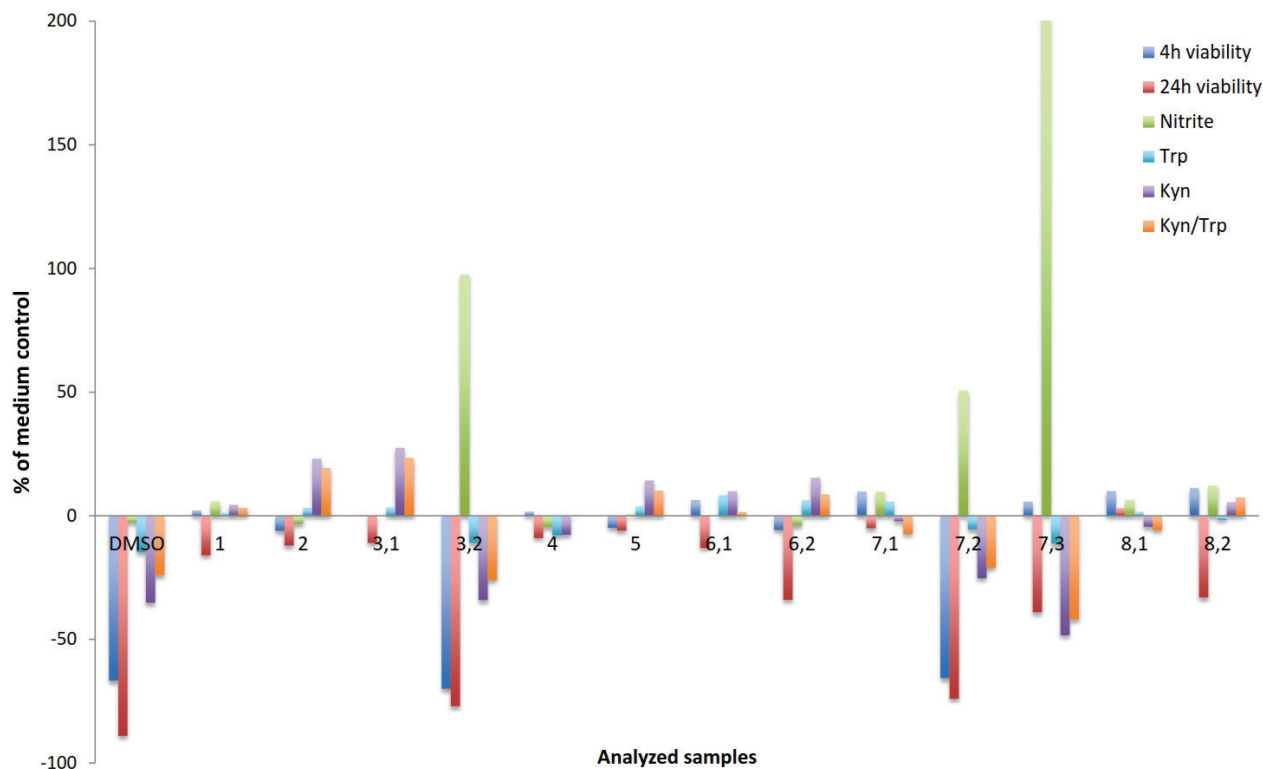


Figure 3: Changes in cell viability %, nitrite, tryptophan and kynurenine levels.

of cells exposed to sample extracts. Nitrite levels were determined after one, two and four hours of exposure to tested material. Results are given in Table 2. Significant increase in nitrite levels were obtained for samples 3.2, 7.2 and 7.3. Since changes in Trp and Kyn levels are also considered as inflammation markers, we also measured Trp and Kyn levels in the supernatant of cells exposed to sample extracts for one, two and four hours. Table 2 presents Trp and Kyn levels obtained for four hour exposure only because the levels of Trp and Kyn did not change significantly after treatment for one and two hours.

Discussion

There are many post marketing surveillances that monitor the biocompatibility of marketed medical devices. Although some of these products are thought to be biocompatible and are licensed and marketed they are in fact not compatible with the biological system [12, 13]. That is why the goal of this study was to check the biocompatibility of marketed mask products.

The ISO 10993 standard has been prepared to ensure the safety and biocompatibility of marketed medical

devices. For surface devices on the skin, the standard recommends to evaluate their cytotoxicity, sensitization and irritation capacity [4]. Concerning the cytotoxicity, the standard claims that a device can be considered as not cytotoxic if the highest concentration of its extract (100%) allows a cell viability of over 70% after an exposure for 24 hours [9]. Preliminary investigations carried out indicated that samples 1, 2, 4 and 5 do not show any cytotoxicity while samples 3, 6, 7 and 8 were found to be cytotoxic. Since the masks found to be cytotoxic are composed of non-woven material, elastic strip (3, 6, 7, 8) and sponge (7 only), we further separately analyzed the cytotoxicity of each of these materials. The non-woven materials did not show any cytotoxicity (samples 3.1, 6.1, 7.1, 8.1) suggesting that non-woven materials are appropriate for the development of medical devices. However, extracts obtained from elastic straps or sponge constituting the masks were found to be cytotoxic since cell viability dropped to around 30% for samples 3.2 and 7.2 even after four hours of exposition and around 60% for samples 6.2, 7.3 and 8.2 (Figure 2). These results point out the importance of testing devices as a whole before allowing them to be marketed since minor constituents can also be cytotoxic.

When evaluated for the inflammatory response that the mask samples induced, we first looked for the changes in nitrite levels in L929 cells. Indeed, increase in nitrite levels is a well-known indicator of inflammation for these murine fibroblasts [14-17] and this increase has been demonstrated to be tetrahydrobiopterin dependent [18, 19]. Samples 3.2, 7.2 and 7.3 clearly demonstrated an inflammatory response since a 1.5-fold, 2-fold and a 4-fold increase in nitrite levels were obtained for samples 7.2, 3.2 and 7.3 respectively after four hours of exposure. These samples were also found to be cytotoxic, suggesting a possible correlation between the inflammation and cytotoxicity. Indeed, NO plays an important role in the regulation of cell mediated immune response but massive NO production can induce apoptosis and thus cell death [21, 21]. Additionally, oxidative DNA damage can be generated during immune response by NO production [22] which would lead to an activation of the apoptosis process and cell death.

The increase in the Kyn/Trp ratio is also considered a hallmark of inflammation. Indeed, Trp is an essential amino acid with three key roles in the cell: it is used as a building block in the protein synthesis and is also the precursor of two important intermediates that are serotonin and niacin [23]. Niacin, a key cofactor of many proteins, is obtained *via* the Kyn pathway where Trp is first converted into Kyn (the rate limiting step) and then to three other intermediates before being transformed into the final compound. As the Kyn pathway has been found to be upregulated in the inflammation process, an increase in the Kyn/Trp can also be an indicator of inflammation [24]. Therefore, we also monitored the Trp and Kyn levels in cells exposed to the sample extracts. Our results indicated a decrease in Trp levels for samples with which cytotoxicity was also observed (sample 3.2, 7.2, 7.3) (Figure 3). However, no increase in Kyn levels was recorded for these samples and therefore the Kyn/Trp ratios did not show any increase neither, even though increase in nitrite levels were pointing the existence of an inflammatory response. These findings can be explained by the important cytotoxicity of these samples. The cell death process activated by the cytotoxic samples upregulates many pathways that require the synthesis of proteins which will cause a decrease in tryptophan levels [25]. The similar decrease obtained for Kyn levels suggest that the activation of IDO is not significant when compared to the use of Trp in protein synthesis. Therefore, monitoring the Kyn/Trp ratio to detect inflammation seems to be unsuitable for samples that are too cytotoxic and also unsuitable to observe an increase in IDO activity, it would be necessary to work in non-cytotoxic concentrations.

Furthermore, melatonin, a hormone that is synthesized from serotonin for which Trp is the precursor, has been reported to have anti-inflammatory and antioxidant effect [26]. Thus, its use in case of an oxidative stress or inflammation would imply an increase of its synthesis and thus a decrease in Trp levels that may not affect Kyn levels [27, 28].

Also, Kyn has been recently shown to demonstrate a potential role on immunomodulation and wound healing by suppressing the proinflammatory cytokines [29], a finding that can also explain the observed decrease in Kyn levels in samples for which an increase in nitrite levels was obtained (3.2, 7.2, 7.3).

Given these results, the consumer can expect to develop an inflammatory reaction to surgical masks even throughout a surgical operation since increases in nitrite levels are significant even for only 1 hour of exposure. Moreover, masks may induce inflammation more easily in vulnerable patient with infectious diseases or oncology patients when used for protection against surrounding risks [30]. This may lead to further potential risks such as irritation and hypersensitization.

In this study, the evaluation of the biocompatibility of surgical masks was carried out on 3 different groups of surgical masks. A total of 8 masks were analyzed. Cytotoxicity was assessed via the MTT cell viability assay on L929 murine fibroblasts as suggested by the ISO 10993-1 standard. Inflammation was monitored by measuring nitrite, kynurenine and tryptophan levels. The results obtained even with this limited number of samples indicated that these widely used disposable medical devices induced a surprisingly high rate of cytotoxicity and inflammation. Thus, this study suggests that monitoring inflammation with cytotoxicity could be valuable to determine the biocompatibility of medical devices, as cytotoxicity has been described to be related to inflammation as already discussed. To evaluate the inflammatory responses, pteridines levels and especially the levels of tetrahydrobiopterin, the essential cofactor for the synthesis of nitric oxide, could also be measured in addition to nitrite levels.

In conclusion, post marketing vigilance against the risk of finished medical devices and routine monitoring of biocompatibility of marketed medical device is crucial to protect public health. Precautions should be taken to reduce possible side effects on consumer.

Conflict of Interest: The authors have no conflict of interest.

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