ORIGINAL ARTICLE

Characterisation of Metallic Chemical Elements Used as Colour Additives in Cosmetic Colour Contact Lenses and Effects on Human Corneal Epithelial Cell Viability *in Vitro*

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ABSTRACT

Introduction: A colour additive is a dye incorporated in contact lenses for cosmetic reasons. The information of the additive's metallic elements and the impact on the human cornea is however limited. We aimed to characterise the metallic elements used as colour additives in cosmetic colour contact lenses (Cos-CCLs) and to investigate their effects on the human corneal epithelial cells. **Methods:** Two contact lens brands, Freshkon (FK) and AirOptix (AO) of three colours (brown, green, grey) were studied. A Field Emission Scanning Electron Microscope equipped with Energy Dispersive X-ray Spectroscopy (FESEM-EDX) was used to characterise the chemical elements used as the colour additives. The findings on the chemical elements were compared between the Cos-CCLs and their respective clear contact lens counterparts. The effects of Cos-CCLs and clear contact lenses on human corneal epithelial cells (HCECs), in vitro, was assessed using an MTT-assay over an 8-hour time period. **Results:** Five key metallic constituents were identified in all Cos-CCLs but were not found in clear contact lenses. The most frequently detected metallic additive was iron. HCEC viability was detrimentally affected more by Cos-CCLs than the clear contact lenses. **Conclusion:** Dissimilar metallic additives combinations are used by manufacturers to achieve the different coloured effects in a range of coloured contact lenses. Vitro data exhibits a detrimental effect of metallic additives on the human corneal epithelial cells. Nevertheless, more real case scenarios are still required to support our toxicity investigation and inference.

Keywords: Colour additives, Cosmetic colour contact lens, SEM-EDX analysis, MTT assay, HCEC viability

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INTRODUCTION

In ophthalmology and optometry, colour additives were used to impregnate cosmetic colour contact lenses (Cos-CCLs) thus changing the appearance or colour of the eyes. There is however a lack of evidence-based information in literature on the evolution and efficacy of incorporating colour additives into contact lenses. The initial idea of applying colour additives to the body matrix of a contact lens was proposed by Fick who highlighted the potential prosthetic benefits associated with the use of painted contact lenses (1). There are many clinical reasons associated with the incorporation of colour additives into contact lenses such as assisting with lens handling and identification, improving the cosmesis of eyes with corneal scars and disorganised anterior segments and the management of glare and photophobia. However, they are increasingly being marketed as colour contact lenses purely for cosmetic

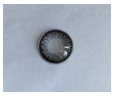
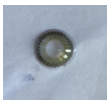
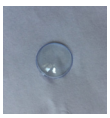
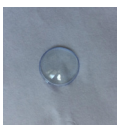
reasons.

Cos-CCLs comprise of a central colour free optical zone and a peripheral coloured surround. The perception held by the general public and given by the fashion industry is that Cos-CCLs were simply a cosmetics accessory by changing or enhancing the eye colour (2-3). In Asia, the popularity of Cos-CCLs has increased particularly among teenagers and this is fuelled by the fact that lenses are widely available online (2-3). Consequently, the associated risk implications for ocular health has become a major concern in many countries (4-7).

The lack of information regarding the chemical elements used to achieve the coloured effect in Cos-CCLs is alarming as there are now a wide range of colours and brands available from a variety of manufacturers and suppliers. Information about the chemical elements used to produce colour effects in Cos-CCL products should be published on guidance information leaflets, contact lens containers and packaging but this is not mandatory for manufacturers.

The use of metallic elements as colour additives are common in various products like foods and cosmetics. Thus, their effects pertaining to toxicity issues to human tissues have become a major concern (8-9) where several laboratory studies have identified metallic elements including iron, titanium and aluminium in them (10-11). We believe that it is not only important to specify which elements are used to create the coloured effects in individual contact lenses but also to determine if the addition of metallic elements create risk of adverse events. On top of that, the risk pertains to all Cos-CCLs regardless of colour range or brand should also be ascertained. This study will systematically characterize the chemical elements used as colour additives in the

Table I Information on eight test samples: the cosmetic colour contact lens samples with their respective clear contact lens counterparts

Sample	Product Name	Colour	Physical Colour Appearance	Contact Lens Material	Water Content (%)	Diameter (mm)	Base curve (mm)	Refractive Power (D)	Brand	Colour Additive Information
FK1	FAE	Brown		Etafilcon A	58	14.2	8.5	- 0.50	FK	NIL
FK2	FAE	Grey		Etafilcon A	58	14.2	8.5	- 0.50	FK	NIL
FK3	FAE	Green		Etafilcon A	58	14.2	8.5	- 0.50	FK	NIL
FK4	58F	Clear		Etafilcon A	58	14.2	8.5	- 0.50	FK	N/A
AO1	AOC	Brown		Lotrafilcon B	33	14.2	8.6	- 0.50	AO	NIL
AO2	AOC	Grey		Lotrafilcon B	33	14.2	8.6	- 0.50	AO	NIL
AO3	AOC	Green		Lotrafilcon B	33	14.2	8.6	- 0.50	AO	NIL
AO4	AO	Clear		Lotrafilcon B	33	14.2	8.6	- 0.50	AO	N/A

Note: FK – Freshkon; FAE – Freshkon Alluring Eyes; 58F – Freshkon 58; AO – Air Optix; OC – Air Optix Colour; NIL – no information listed; N/A – not applicable [Source: Products' ackages]

two different brands and modalities of lenses and assess their effects on human corneal epithelial cell viability in vitro.

MATERIALS AND METHODS

Contact Lens Samples

Contact lens samples used in this study were purchased from optometric premises in local markets in Selangor, Malaysia. Two brands of contact lenses, Freshkon (FK) (Oculus) and AirOptix (AO) (Alcon), were chosen as representative of high and low water content materials respectively, as these lens types are frequently considered as options in prescribing of contact lens. They were purchased randomly in different optometric premises to ensure they were from different manufacturing batches. Table I summarises the parameters of the samples as described on their packages. Three groups of colours were selected for each brand: brown, green and grey. These colours were selected due to the consistency of availability of the colours in both brands. For each brand, one group of clear contact lens samples was included as control and reference.

Characterisation of Chemical Element of the Contact Lens Samples

A field emission scanning electron microscope (JSM 6701F) equipped with an electron dispersive X-ray probe (FESEM-EDX) was used in this study (9). The instrumentation used was selected because of its ability to identify chemical elements across a broad spectrum of the periodic table. The theory relies on the emission of specific X-ray spectra, which characterises particular chemical elements when bombarded with a focused beam of electrons from the FESEM's electron probe. Elements with an atomic weight lighter than beryllium (Be) are not identifiable using this technology.

The contact lenses selected for analysis were divided into quadrants, using a fresh clean razor blade, surface dried, and fitted onto the scanning electron microscope's stubs. For each contact lens, two surface orientations (front and back surface) were prepared for this analysis (9). The stubs containing samples were placed carefully into the FESEM chamber with the orientations under examination and clearly noted. The characterisation of chemical components that constituted the colour additives was performed using EDX analysis.

Normal Human Corneal Epithelial Cells (HCEC), Media and Reagents

HCECs were obtained from EpiGRO (Merck, Germany). Upon receiving the cells, they were cryopreserved in a liquid nitrogen vapor phase at passage two. In this experiment, data collections using the HCECs were carried out between passage three and passage five. Beyond passage five, it was observed that cells have become senescent. The culture media used to grow the human corneal epithelial cells consisted of basal media and supplemented growth factors; EpiGRO™ Human Ocular Epithelia Complete Media Kit (Merck, Germany). The growth factors were from Gibco (Thermo Fisher Scientific, USA), comprised of L-Glutamine, EpiFactor O, Epinephrine, EpiFactor P, rh Insulin, Apo-Transferrin and Hydrocortisone Hemisuccinate. Antibiotics (Penicilin-streptomycin) and dissociation reagent (TrypLE). Dissociation of the cells was neutralised with trypsin neutralisation solution (TNS) (Lonza, Switzerland). Cells were counted using trypan blue (Merck, Germany). The cell viability assay [3-(4,5-dimethylthiazal-2-yl) – 2,5-diphenyl tetrazolium bromide] powder, abbreviated as MTT and dimethyl sulfoxide (DMSO) from Merck (Merck, Germany).

Cell Culture

Human corneal epithelial cells (HCECs) were carefully thawed in a 37 C water bath. The vials containing HCECs were wiped dry and sprayed with 70% ethanol before being brought into the biosafety cabinet. About 5mL of the completed media was then added into T-25 culture flasks. The HCECs were inoculated into the T-25 culture flasks containing completed culture media at a density of 5.0 x 103 cells/cm^2 and were then incubated in 5% CO_2 / 95% humidity at 37°C overnight.

The next day, the culture media was carefully aspirated from each culture flask. The HCECs were washed with 2mL phosphate-buffered saline to remove any residual DMSO. The phosphate-buffered saline was then aspirated from the culture flasks. About 5mL of fresh completed culture media was added to the culture flasks and changed every other day until HCECs reached 80% confluency. HCECs morphology and confluency were assessed using an inverted phase-contrast microscope every day for 4-5 days (reaching 80% confluency). HCECs were incubated in 5% CO_2 / 95% humidity at 37°C throughout the culture process.

Upon reaching 80% confluency, the culture media was aspirated from each culture flask and HCECs were detached by treating them with 2mL of TryPLE for 10 minutes in 5% CO2 / 95% humidity at 37°C. Cell detachments were observed through the inverted-phase contrast microscope. HCECs detachments were then neutralised with 2mL of TNS. The mixture was centrifuged at 150 x g for 5 minutes. The supernatant was then discarded, and the resultant cell pellet was resuspended with 1mL of fresh culture media before the HCECs were seeded either for subculture or treatment.

Cell Treatment

For treatment, HCECs were seeded in 24-well plates at a density of 1.0 x 105 cells per well. The plate was incubated in 5% CO2 / 95% humidity at 37 C for 24 hours to allow cell attachment. After 24 hours, the culture media was carefully aspirated from each well. 200µL of fresh culture media was then added into each well before the contact lens samples were placed into each designated well except one which was designated

as an untreated control well where a contact lens had not been introduced. All contact lens samples were carefully introduced into each well using a pair of clean and sterile tweezers. The back surface of each contact lens was inverted within its designated cell. Another 200µL of fresh culture media was added on top of each contact lens sample before the plate was incubated for 8 hours in 5% CO_2 / 95% humidity at 37°C, simulating a normal recommended wearing period.

MTT-assay

After eight hours of cell treatment, the contact lens samples were carefully removed from each well using a pair of clean and sterile tweezers. The 200µL of freshly prepared MTT solution (20mg/mL) was then added into each well and incubated in 5% CO_2 / 95% humidity at 37°C for four hours. After that, the mixture of MTT solution and culture media from each well was carefully aspirated, leaving violet formazan crystals formed by the viable cells. The 300µL of DMSO solution was subsequently added into each well to solubilise the violet formazan crystals which now has different colour intensities. Finally, those colorimetric intensities were measured with a spectrometry reader using a filter absorbance of 570nm wavelength.

Data Analysis

Descriptive data of the characteristic chemical elements from both surface orientations of the contact lens samples was obtained from the EDX spectroscopy. Different characteristic chemical elements between Cos-CCLs and their respective clear contact lens counterparts within their own brand confirmed that the chemical elements belonged to the colour additives of Cos-CCLs (9). Measurements of colorimetric intensities represented the estimation of viable cells left after the treatment. The denser the colour intensity, the higher the number of viable cells estimated in the well. The colorimetric intensity data obtained was analysed using the Statistical Package of Social Sciences (SPSS) version 21.0.

The descriptive data were presented as mean ± standard deviation from the triplication of data in three independent experiments. The statistical analysis comparing mean values that represent the effects of treatment across wells was performed using a one-way ANOVA with a Tukey HSD post hoc comparison test. The mean value comparison between treatment wells of $p \leq 0.05$ was predetermined as the criterion for statistical significance. Later, the percentage of cell viability from each treatment well was calculated relative to the untreated control well with the assumption that the untreated control well contains 100% viable cells. The equation (1) for the calculation was described as follows:

$$\text{Cell vialibility (\%)} = \frac{\text{(absorbance of the treated well)}}{\text{absorbance of the untreated control well}} \times 100\% \qquad \text{Equation (1)}$$

RESULTS

Chemical Elements Characterised by SEM-EDX Analysis on Two Types of Commercially Available Cos-CCLs

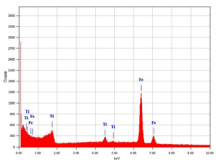
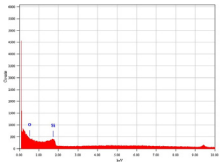
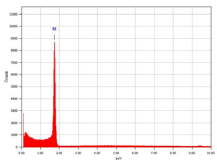
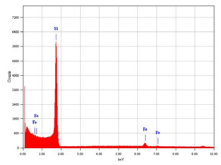
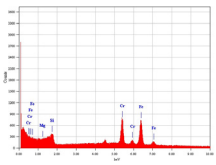
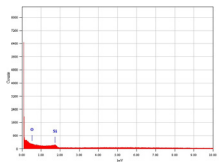
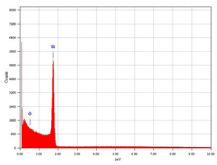
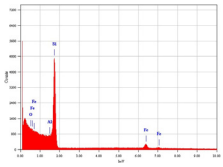
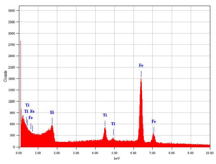
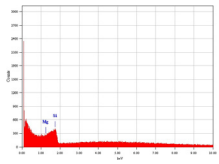
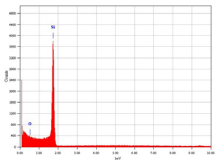
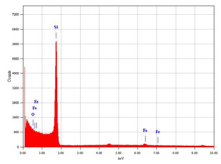
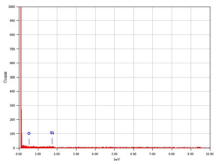
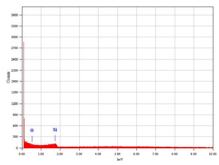
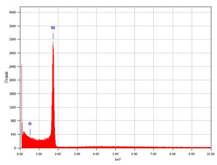
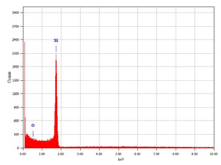
Characterisation of the elements by EDX spectroscopy is based on measurement of the X-ray energy released during electron transition from a higher to a lower energy level, when the samples are bombarded with the electron beam. The X-rays emitted from the sample atoms are characteristic and specific in energy and can specifically identify the constituent elements. The spectral graphs of the elemental analysis for each contact lens sample are shown in Table II.

On the spectral graphs, the X-axis represents the characteristic X-ray energy released by electrons from a particular atomic shell. The characteristics illustrated on the graphs are specific for each element all of which have been labelled as abbreviation letters on the graphs. Some of the elements may appear to have multiple peaks along the X-axis. Individual peaks are dependent not only on an element's atomic number but also according to which atomic shell involved in the electron transition for that particular element. The Y-axis quantifies the X-ray energy intensity and is strongly dependent on the amount of each element present in the sample as well as the settings of the SEM used by the operator. For illustrative purposes, and in order to clearly demonstrate the relative proportions of differing elements within individual lenses, we have chosen to use different Y axis ranges for different lenses.

The results clearly show that there were chemical elements identified in all the Cos-CCLs which were not found on control clear contact lenses, indicating that these are likely to be the chemical elements introduced as colour additives. The differences in the elements would also tend to indicate that combinations of specific elements have been used to achieve different colour effects. The characteristics of the elements in terms of their percentage composition on each surface of each sample are highlighted in Table III for the five metallic elements identified (Iron, Titanium, Chromium, Aluminium and Magnesium). None of these elements were found in the clear lenses.

Iron element has been identified in all coloured lenses of both brands. In clear contact lens samples of both brands, silicon represents 100% composition of the elements identified on the matrix. Our findings clearly demonstrate that with the exception of the grey lens, the chemical

Table II Spectral graphs of the elemental analysis of the FK and AO contact lenses

Sample	Product Name	Colour	Front Surface	Back Surface	Sample	Product Name	Colour	Front Surface	Back Surface
FK 1	FAE	Brown			AO 1	AOC	Brown		
FK 2	FAE	Grey			AO 2	AOC	Grey		
FK 3	FAE	Green			AO 3	AOC	Green		
FK 4	58F	Clear			AO 4	AO	Clear		

Note: FK – Freshkon; FAE – Freshkon Alluring Eyes; 58F – Freshkon 58; AO – Air Optix; AOC – Air Optix Colour.
Abbreviations: Si, silicon; Fe, iron; Ti, titanium; Cr, Chromium; Mg, magnesium; O, oxygen]

Table III: Chemical elements and their percentage composition identified in the contact lens samples characterized by the FESEM-EDX

Color	Chemical elements (mol %)	
	FK	**AO**
Clear (control)	Silicon (100.00; ***100.00***)	Silicon (100.00; ***100.00***)
Brown	Silicon (2.30; ***100.00***), *Iron (96.18), *Titanium (1.52)	Silicon (100.00; ***87.15***), *Iron (***12.85***)
Green	Silicon (2.36; ***100.00***), *Iron (85.57),*Chromium (7.72), Magnesium (4.34)	Silicon (100.00; ***74.62***), *Iron (***24.59***), *Aluminium (***0.79***)
Grey	Silicon (1.41; ***74.56***), *Iron (91.78),*Titanium (6.81), *Magnesium (***25.44***)	Silicon (100.00; ***86.98***), *Iron (***13.02***)

FESEM-EDX, Field emission scanning electron microscopy equipped with energy dispersive X-ray analysis; FK, Freshkon; AO, AirOptix. Boldface and italicized numbers in the brackets indicate mol % found on the back sides of the contact lenses; the non-boldface and non-italicized numbers indicate mol % found on the front sides of the contact lenses [notes: x-axis represents the elemental finding (identifiable by the characteristic x-ray energy value) whereas y-axis represents the elemental counts].
*Chemical elements exclusively identified on the Cos-CCLs with the respective clear CLs used as a control reference.

elements of colour additives used in FK Cos-CCL samples appear only on the front surface. Chemical elements of colour additives used in the AO lenses however appear exclusively on the back surface. In the case of the FK grey Cos-CCL, whereas the majority of chemical elements used to create the colour effects appear on the front surface of the lens, magnesium (25.44%) was found on the back surface. From the table, the percentage composition of the chemical elements used in different lenses

varied between samples with iron contributing the highest percentage as a colour additive embedded in all Cos-CCL samples of the FK brand (ranging from 85.57% to 96.18%). This exceeds the main component of the contact lens matrix which is silicon. In contrast to the FK Cos-CCL samples, the percentage of iron characterised in the AO Cos-CCL samples did not exceed the silicon component. However, their percentage composition was considerably higher as compared to the other elements characterised as colour additive elements.

Effects of Cos-CCLs on HCEC Viability *in Vitro*

In this study, the results of the colorimetric absorbance readings obtained from the triplication of experiments are expressed in mean (± SD). As mentioned earlier, the colorimetric absorbance readings represent the estimation of viable cells. The higher the reading, the greater the number of estimated viable cells. Table IV shows the colorimetric readings and the calculated percentage of HCECs viability. ANOVA testing shown significant mean differences between treatment groups for both contact lens brands ($p<0.05$).

The threshold colorimetric reading obtained from the untreated control group (contact lens samples were not introduced), was 0.050; indicating 100% HCECs viability. Treating the HCECs culture to the FK clear contact lens samples reduced the HCECs viability slightly from the threshold value to 0.049 ($p=0.70$). Significant reductions of HCECs viability from the threshold value was however observed when all three groups of coloured contact lenses were treated on the cells ($p<0.05$). Brown colour has reduced the human corneal epithelial cells viability to 0.045, green colour to 0.045 and the highest reduction was caused by the grey colour to 0.044. Post-hoc data analysis also showed significant difference of the HCECs viability between all three coloured contact lens groups as compared to the clear contact lens: brown ($p=0.004$), green ($p=0.002$) and grey ($p<0.05$).

Treating the AO brand contact lenses to the HCECs culture resulted in similar viability trends. Although clear contact lenses reduced HCEC viability to 0.049 from the threshold untreated group, the difference between them was not significant ($p=0.85$). HCECs viability has further reduced to 0.044 with brown coloured contact lens treatment, 0.040 with grey contact lens treatment and 0.039 with green coloured contact lens treatment. The differences in HCECs reduction were also significant between all three coloured groups and their clear counterparts ($p<0.05$). Fig. 1 and Fig. 2 depicted the findings in percentage HCEC viability with Tukey post-hoc pairwise analysis. HCECs viability was notably lower for the coloured contact lens groups of the AO brand as compared to the FK-coloured contact lens treatment groups specifically for the green and grey colours, in the range of 9% - 11%. There was only a very small percentage difference of the HCECs viability between clear contact lenses for both groups at only 1%.

Table IV: Colorimetric absorbance readings and their respective estimated HCEC viability

Contact lens brand	Treatment	Colorimetric absorbance reading / mean ($x10^{-2}$) (± SD)	Estimated of the viable cells / (%)	ANOVA
FK	Untreated (control)	5.05 (± 0.06)	100.00	
	FK4	4.91 (± 0.06)	97.00	
	FK1	4.52 (± 0.16)	89.00	p < 0.001
	FK2	4.49 (± 0.06)	89.00	
	FK3	4.36 (± 0.08)	86.00	
AO	Untreated (control)	5.05 (± 0.06)	100.00	
	AO4	4.93 (± 0.06)	98.00	
	AO1	4.39 (± 0.20)	87.00	p < 0.001
	AO2	3.95 (± 0.008)	78.00	
	AO3	3.96 (± 0.04)	78.00	

Note: HCEC – Human corneal epithelial cells; Cos-CCL – cosmetic color contact lens. Results shown were the mean values from the triplication of the experiment, n=3. ANOVA tests were predetermined significant at $p < 0.05$.

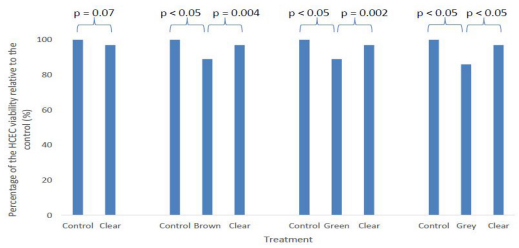


Fig. 1 : Human Corneal Epithelial Cells (HCEC) viability following an 8-hour treatment period with different colours of Freshkon (FK) contact lens samples. P-values showing post-hoc Tukey pairwise comparison between groups.

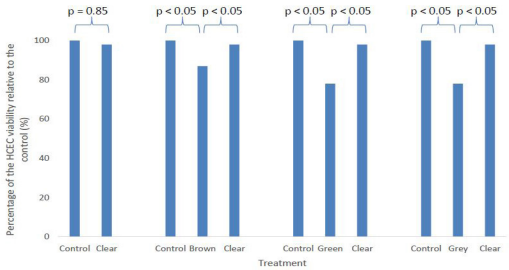


Fig. 2 : Human Corneal Epithelial Cells (HCEC) viability following an 8-hour treatment period with different colours of AirOptix (AO) contact lens samples. P-values showing post-hoc Tukey pairwise comparison between groups.

DISCUSSION

This study provides a direct comparison of the chemical elements identified in a selection of Cos-CCLs and compares the results with those recorded from clear comparators. Chemical elements that were found solely in the Cos-CCLs and not found in the respective clear contact lenses are assumed to be the colour additives. All chemical elements found were from the metallic group with iron found in all Cos-CCL samples. This confirms the results published in a previous study (8). It would thus appear that iron serves as the core element used as a colour additive in a range of Cos-CCLs. This study also confirms that a variety of other chemical elements were used in combination as colour additives in Cos-CCLs to achieve the desirable colour appearance.

Two different strategies have been identified and used to incorporate the colour additives in the two different contact lens brands in this study: FK brand applies colour to the front surface, while the AO brand applies colour to the back surface. Incorporating colour additives to the surface of contact lenses has been demonstrated to increase contact lens surface roughness (12) which may in turn cause disruption to corneal epithelial functions which consequently causes irritation and discomfort at the ocular surface (13-14). Disruption to corneal epithelial functions can cause not only ocular irritation, but an increased risk of microbial infections resulting from a breakdown in the cellular barrier function (15-18). In addition, the roughness of the Cos-CCLs surfaces may promote microbial adhesion and subsequently increase the risk of corneal infection (19-20).
Having identified the chemical elements of the colour additives used in a selection of Cos-CCL samples containing various metallic elements, we sought to study the effect these elements may have on corneal epithelial tissue. Employing an MTT assay, we have demonstrated that the metallic constituents used in Cos-CCLs can compromise the human corneal epithelial cells in vitro, over what simulates an 8-hour wearing time. This approach was taken to represent normal contact lenses wearing patterns. MTT-assays were used as a routine method to investigate tissue viability and substance toxicity (21-23). MTT method is useful to measure cell proliferation and cytotoxicity (24-25). The purple colour dye can be used to measure cytotoxicity (26-28). Growing numbers of cells deepen purple colour (26-28). The reduction of cell metabolic activities in the MTT assay can be captured by spectrophotometry as an index of cell viability (24-28).

It was also demonstrated in this study that HCECs viability was significantly reduced in all Cos-CCL groups when compared to the untreated HCECs and respective clear contact lens counterparts. Thus, the findings were somewhat as expected as the presence of metallic elements as colour additives in Cos-CCLs should not be underestimated knowing that metallic elements are known to catalyse toxic damage in human tissues (29-32). Although most metallic element toxicity in tissue occurs in the organs that acquire their source from the blood-stream where they are transported by the protein transferrin, they may also get internalised into cells through other poorly defined mechanisms (33). Hence, the application of Cos-CCLs which contain metallic elements might introduce a potential source of damage to corneal epithelial cells through direct contact of the products, even though the cornea is an avascular tissue.
The elements of colour additives were found in our study on the surface of the FK cluster (specifically on the front surface because the images of the colour additives were well captured in this orientation as compared from the back surface of the same samples), while the images of colour additives were not clearly seen from both surfaces of the AO cluster. This suggests that the elements were located in the middle (sandwich technology). As the chemical elements were identified on the front surface of the FK cluster, the composition of the colour additives elements predominated the main element of the body matrix of the CLs made up of silicon (considering the surface area covered by the coloured zone is larger as compared to the cleared zone in any type of Cos-CCLs). However, the composition of colour additives in the AO cluster were seen lower relative to the main element of the body matrix because they were located in

the middle, covered by the main body matrix of the CLs. We have also shown that two different brands of Cos-CCLs have different effects on the HCECs viability which was lower in the AO Cos-CCL treatments as compared to the FK Cos-CCLs. These differences in the HCECs viability could be attributed to multiple factors. The positioning of the colour additives being one of them. In our study, SEM findings demonstrated that colour additives were found on different lens surfaces for each brand. In the case of the FK lenses, colour additives were on the front surfaces whereas they were on the back surface of the AO lenses. As the location of colour additives are on the back surface of the AO Cos-CCL samples, we speculate that the potential toxic effects on corneal epithelial cells may be more detrimental. We also introduced the contact lens samples with their back surface oriented in direct contact with the HCECs culture. Thus, AO Cos-CCLs which have the colour additives on the back surface affected the HCECs viability more extensively. The detrimental effects of epithelial cell viability in this study are limited to short-term in-vitro investigations on the brands.

CONCLUSION

Cos-CCLs embrace distinctive metallic elements that are not present in clear contact lenses and consequently caused significant reductions of corneal epithelial cell viability in vitro. Dissimilar metallic additives combinations have been used by manufacturers to achieve the different coloured effects in a range of coloured contact lenses. Based on in vitro data collected, these metallic additives have a detrimental effect on the human corneal epithelial cell, but more real case scenarios are required to support our investigation and to make more affirmative inferences.

ACKNOWLEDGEMENTS

This work is supported by Universiti Teknologi MARA [600-IRMI/MyRA 5/3/GIP (041/2017)]. The authors would like to thank the Imaging Centre, Faculty of Pharmacy, UiTM Puncak Alam Campus for providing technical support during our work with the Field Emission Electron Microscope (FESEM) JSM-6701F with the energy dispersive X-ray (EDX) spectroscopy attachment (JEOL) and Institute of Molecular Medical Biotechnology (IMMB), UiTM Sungai Buloh Campus for providing technical support during our work with the human cell culture laboratory.

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