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RESEARCH ARTICLE

Effects of Laminar Flow Ultraviolet Sterilization Protocol on *Cryptosporidium parvum* Oocysts and Determination of These Effects with Vital Dyes

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Summary

Cryptosporidiosis is a globally widespread protozoan disease with zoonotic character. Protection and control bear great importance for combating the disease that may cause serious economic losses. At this point, UV (ultraviolet) may be used to inactivate the oocysts those are infectious form of the disease. In this study, determination of the effect of UV sterilization process used in routine cell culture laboratory sterile cabins (laminar flow) on *Cryptosporidium parvum* oocysts and the efficiency of vital dyes (DAPI, PI) in the demonstration of the changes made by UV applications in question on oocysts is aimed.

Keywords: *Cryptosporidium parvum, Ultraviolet, Vital dye*

Cryptosporidium parvum Oocystleri Üzerine Laminar Flow Ultraviole Sterilizasyon Protokolünün Etkisi ve Söz Konusu Etkinin Vital Boyalarla Belirlenmesi

Özet

Cryptosporidiosis dünya genelinde yaygın olarak görülen, zoonotik karakterde bir hastalıktır. Önemli ekonomik kayıplara neden olabilen hastalıkla mücadelede korunma ve kontrol büyük önem arz etmektedir. Bu noktada, hastalığın enfeksiyöz formu olan oocystlerinin inaktivasyonunu sağlamak amacıyla UV (ultraviolet) kullanılabilmektedir. Bu çalışmada, rutin hücre kültürü laboratuvarları steril kabinlerinde kullanılan UV sterilizasyon işleminin *Cryptosporidium parvum* oocystleri üzerindeki etkisinin ve UV uygulamasına bağlı olarak oocystlerde meydana gelen değişikliklerin belirlenmesi konusunda vital boyaların (DAPI, PI) etkinliklerinin belirlenmesi amaçlanmıştır.

Anahtar sözcükler: *Cryptosporidium parvum, Ultraviole, Vital boya*

INTRODUCTION

In cryptosporidiosis being a globally widespread disease with zoonotic character and lacking an efficient specific treatment may spread millions of oocysts with infected host feces. Taking in a small number of these oocysts which are very tiny and resistant against disinfectants and different environmental conditions may lead to infection. That is why protection and control has a special importance for combating the disease [1]. Water is the main source of contamination since oocysts may survive for months, in terms of the agent sensitive against drying. Because the routine water purification filters and chemicals are mostly ineffective in eliminating the agent studies on different methods of water purification has gained intensity. At this point, UV comes into prominence because of some special advantages [2]. UV applications may also be benefited in laboratories

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to sterilize the working places and UVC (254 nm) is a commonly used for this purpose [3,4].

It is possible to consolidate the effect of UV, a non-ionized radiation type, on live organisms in two groups. The first one is "direct effect" [3,4]. This damage type appears with DNA breaks caused by absorbed radiation or dimmers causing deterioration in DNA transcription and replication. The other is the "second effect" originating from radicals formed in and around affected cells and some proteins synthesized by the affected cells [3,5]. Generally, this effect may take a more dominant form than direct DNA damage in cell response forming against UV [6]. The leading one of the aforementioned radicals is the oxygen atom that is charged by losing electron. To be stable these atoms attach to the negatively charged structures around like DNA and spoil the electrical formations carrying vital importance for them. Furthermore, after UV applications, some of the molecules released in a few days related with the doses may lead the cell to apoptosis. In the basis of this strategy, there lies the effort to refrain the cell from cancer formation risk which is a kind of delayed effect of radiation [6-9].

In this study, demonstrating the effect of laminar flow UV disinfection used in standard cell culture laboratory on *C. parvum* oocysts and finding out the success of vital staining methods in determination of the effects in question is aimed.

MATERIAL and METHODS

Oocyst Preperation: In the study, a calf genotype of *C. parvum* was used. Oocysts were obtained from the faeces of an experimentally infected calf 4 months before use and stored at +4°C in phosphate buffered saline (PBS) containing penicilline (100U/ml PBS), streptomycine (0.1 mg/ml PBS) and amphotericin B (2.5 µg/ml PBS) which was changed monthly.

UV Application: In the study, *C. parvum* oocysts, produced in calf through experimental infection under laboratory conditions and kept in antibiotic and anti-mycotic supported PBS at +4°C after purification, were used. To be able to demonstrate the reaction of the aforementioned oocysts against UV sterilization, laminar flow (HERAsafe, K 12, Kendro, Hanau) UV sterilization system was used. For the process to be performed UV application protocol used for sterilizing the cabinet was taken as the basis. To the centre part of the sterile cabinet which has two germicidal fluorescent on each side, oocysts in equal distance to UV (UVC) sources, in an open glass container, in 5 ml PBS (10^5 oocyst/ml) were placed. The mechanism was prepared for all trails as not to pass PBS depth by 1 cm and bilateral UV sources may see oocysts directly from PBS surface. The application period of UV is determined as 30 minutes as indicated in the user's manual of the device.

DAPI-PI Staining: After the process the sample was separated into two groups. One of the groups was taken into an incubator fixed at +4°C whereas the other was taken into an incubator fixed at +24°C. Just after the UV process and in the following 24, 48, 72 and 96 h to demonstrate the vitality changes occurring in oocysts, 4',6-diamidino-2-phenylindole-dihydrochlorid (DAPI, min 98%, AppliChem) and propidium iodide (PI, minimum 95% HPLC, SIGMA) were used. In the application of the method and for evaluation of the results the protocol defined by Campbell et al.[10] was followed. In each trial at least 100 oocysts were counted and the corresponding ratios of data were determined. The ones giving bright red fluorescent under green filter were evaluated as PI(+), the ones giving nucleus based or general blue fluorescent under UV filter block as DAPI(+), and the oocysts those are not giving any fluorescent reflection but showing normal oocyst structure in interference contrast as DAPI(-)PI(-). Same procedure were performed in the negative control groups those were not applied UV and hold in same conditions with the trial groups.

The data were analyzed using the Kruskal Wallis Test. P values of 0.001 or lower were considered significant.

RESULTS

Vital dye applications to the oocyst groups resulted in an average 7.2±0.3% PI (+), 14.8±0.5% DAPI (+) in control group, 19.2±0.4% PI (+), 91.4±0.6% DAPI (+) right after UV application and end of the incubation (at 96th h), 73.1±0.9% PI (+), 93.1±0.4% DAPI (+) in the group kept at 4°C and 100.0±0.0% PI (+), 100.0±0.0% DAPI (+) in the group kept at 24°C. Detailed results of the study are given in *Table 1*. Though, the data obtained from the vital staining done simultaneously directed towards control groups kept at the same conditions with trial groups were similar to main stock.

DISCUSSION

It was reported that UV applications cause diminished infectivity and reduced resistance against heat, change in shape and motion, lagging in division and excitation, change in contractile vacuole efficiency, restriction in perspiration, loss against hydrostatic pressure and death in protozoa, all of which correlated with UV dose [5]. However, with the studies done with *C. parvum* it was

Table 1. *DAPI-PI values of oocysts obtained at different times held in different temperatures after UV application (X±Sx)*
Tablo 1. *UV uygulaması sonrası farklı sıcaklıklarda tutulan oocystlerle ilgili DAPI-PI verileri (X±Sx)*

Conditions of the Applications	**PI (%)**		**DAPI (%)**	
Before UV application	7.2±0.3		14.8±0.5	
After UV application	19.2±0.4		91.4±0.6	
After Incubations				
Temperature	4°C	24°C	4°C	24°C
Time				
24 h	46.2±0.7 ^{a}	65.1±0.4 ^{a}	91.1±0.8 ^{a}	96.7±0.4 ^{a}
48 h	50.2±0.5 ^{b}	69.7±0.6 ^{b}	91.7±0.7 ^{a}	97.2±0.2 ^{ab}
72 h	59.0±0.3 ^{c}	97.5±0.3 ^{c}	92.6±0.6 ^{a}	97.5±0.2 ^{b}
96 h	73.1±0.9 ^{d}	100.0±0.0 ^{d}	93.1±0.4 ^{a}	100.0±0.0 ^{c}

^{abcd} *For every group, different letters indicate statistically important differences (P<0.001)*

observed that infectivity could be decreased 99% in low doses (1 mj/cm^2) and in high doses (230 mj/cm^2) excitation could be depressed [11]. It was stated that in the applications performed in germicidal doses (0.8-119 mJ/cm^2) the oocysts are easily inactivated but the inactivation in question may not follow in parallel to dose, whereas in the applications made for water sterilization oocyst intensity, type of water and environmental temperature does not carry any importance [12].

It is reported that some of the damages at the cells caused by UV could be repaired by the help of a serial mechanism [13] and the regeneration in question could be more successful with increased biological activity [14]. However, different doses of UV was applied to oocysts by some researchers [15-17] and afterwards different methods and waiting periods considered to provide regeneration was used but no reactivation finding was reached. As a result it was indicated that the UV applications performed appropriately could be used in the sterilization directed towards this protozoon. In a study [18] made in low and middle wavelength UV, neither dark nor light sensitive reaction was encountered in the oocysts controlled in terms of vitality in cell culture and in dark and light environment after the process for 5 days and at 5°C and 25°C. In our study, no finding was encountered pointing to a reactivation in terms of the vitality parameters that could be demonstrated by vital stains in 96 h.

In terms of putting inactivation or reactivation occurring after UV applications it was reported that cell culture-PCR [17], animal experiments [15,16], vital staining and excystation methods[19] could be used. However, after UV experiments it was stated that vital staining or excystation was not an appropriate method in terms up following vitality, this type of parameters show vitality higher than normal, thus the developing inactivation lower than normal. At this point, it was emphasized that vital dye effect mechanisms are related with membrane permeability but essentially UV provides inactivation about the damages related with DNA [20-23]. It was reported that DAPI could easily pass through healthy cell membrane, tied to double strand DNA formation tightly and in this respect this was an efficient vitality indicator. However, PI can pass through deteriorated membranes thus is accepted as an indicator of the inactivation [10]. In our previous studies about vital dyes it was seen that, basic obstacle was oocyst wall for DAPI wherever healthy sporozoite membrane forms a serious obstacle for PI. In this study a serious increase in PI value, particularly in DAPI value just after irradiation was observed. These values continued in the following days, the related increase were partially slow in oocysts +4°C whereas faster at +24°C and after process 96th h PI and DAPI values of the oocysts held at room temperature were recorded as 100% for both. The data obtained in the study shows that the UV in applied dose causes some damages on oocyst wall and this damage increased rapidly by time especially at high temperature. Especially change occurring in PI shows a similar damage occurred at sporozoite membrane. The obtained data indicates that UV may have a significant effect over some basic structures on oocyst other than nucleus.

The increase in the ratio of DAPI (+) PI (+) in groups, especially in high temperatures, point out the secondary damage effect of UV. The question that could the reactions originating from radicals occurring after UV application [3,5] be accelerated at high temperatures arises from the data. Furthermore, after the application, at least under the scope of the tried temperatures and periods it was observed that there was no reactivation, conversely a chronic vitality loss occurred. But it may be wrong to interpret the increase in DAPI and PI values

by time as vitality decreases by time. Because with the application of UV, all of the oocysts depending on direct DNA destruction have already dead or at least lost their infectivity but in all of them damage increasing sufficient surface permeability could not yet have developed. Afterwards the secondary UV affects starts and the aforementioned damage in oocyst wall and sporozoite membrane could be completed.

In the study, it was concluded that routine laminar flow UV sterilization protocol has a significant effect on *C. parvum* oocysts and this effect could be observed with vital staining techniques from different perspectives but the results could be interpreted carefully and we can not be sure about measuring vitality change depending on UV by this way. In the evaluations the information that especially not with sporozoite vitality of DAPI but related with the permeability on oocyst wall and this two parameters does not always follow each other in parallel should not be ruled out.

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