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INCREASED SENSITIVITY OF APOPTOSIS DETECTION USING DIRECT DNA STAINING METHOD AND INTEGRATION OF ACRIDINE ORANGE AS AN ALTERNATIVE SAFER FLUORESCENT DYE IN AGAROSE GEL ELECTROPHORESIS AND MICRONUCLEUS TEST

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ABSTRACT

Electrophoresis is a common technique used in identification, quantification and purification of nucleic acid fragments. Some troubleshooting faced us in detection of apoptosis through DNA fragmentation assay. In some samples; electrophoretic pattern of extracted DNA has a flam shape nearly like sever necrotic fragmentation. The new method was evaluated in this study to standardize the apoptotic fragmentation detection. The direct incubation of extracted DNA samples which have a flam shape with ethidium bromide (EtBr) and acridine orange (AO) gave better results than the ordinary method of gel staining in apoptotic bands detection which appeared at 180 bp and its multiples on 1.8% agarose gel. Moreover, due to the hazardous effect of EtBr handling, acridine orange was tested in parallel on the same nucleic acid samples as an alternative fluorescent staining dye. Additionally, a novel micronucleus test can be accomplished in parallel with viability investigation by fluorescent dual AO/EtBr method. The obtained results showed obvious micronucleated and other nuclear abnormalities observations. In conclusion, although acridine orange is less sensitive than ethidium bromide staining in case of agarose electrophoresis, its safety, low cost and metachromatic properties should be considered and advisably, acridine orange can be used as an alternative nucleic acids fluorescent dye with increased sensitivity for apoptosis detection using direct incubation method. Acridine orange can also be integrated for micronucleus technique.

Keywords: DNA staining, safer dye, ethidium bromide, acridine orange and micronucleus test.

INTRODUCTION

Nucleic acid electrophoresis is a technique used to separate nucleic acids (DNA or RNA) fragments according to size and reactivity using a viscous medium, agarose gel. Setting up electric field lead to nucleic acids migratation toward the anode, due to their negative charge of the sugar-phosphate backbone. DNA fragments of different molecular weights are visualized using a fluorescent dye specific for nucleic acids, usually, ethidium bromide (EtBr). EtBr is one of the most widely used nucleic acid stains in molecular biology area (Sabnis, 2010). EtBr has been used as a straightforward staining method with sensitivity of 0.29–1 ng of DNA (Sambrook *et al*., 1989; Tuma *et al*., 1999). It is a heterocyclic stain, which intercalates in double and single stranded nucleic acids emitting red fluorescence under UV light (Lillie, 1977; Carmichael and McMaster, 1980; Bruno *et al*., 1996). EtBr is being both highly toxic and a mutagenic material (Waring, 1965 and Ohta *et al.,* 2001). It also caused neural damage in rats (Kuypers *et al*., 2013). Generally, EtBr has a potential risk to laboratory workers and is considered as a carcinogen (National Toxicology Program, 2005). The stability of EtBr compound reveals that how much important is to treat this compound before disposing (Saeidnia and Abdollahi, 2013). In many developing countries, improper disposal of hazardous waste materials can cause harmful hazardous effects if they integrated into environment and contaminate both land and water consequently, to the human consumed items.

Despites the serious toxicity of EtBr, it is still used in some labs because it is considerably less expensive in comparison to other compounds like SYBR®-based dyes. Although the seller companies claim on safety of SYBR®-Green (Green Viewer), there are reports on higher mutagenicity of this compound in comparison to EtBr in the bacterial cells exposed to UV (Saeidnia and Abdollahi, 2013).

An attempt to proceed with an alternative less hazardous fluorescent dye for nucleic acids staining has to be paid an attention and considerable concern. Acridine orange (AO)

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has been used extensively in cytochemistry to monitor flow cytometry, nuclear fragmentation, pH gradients in biological membranes, and for various other applications (Palmgren, 1991; Zelenin, 1993; Hitoshi *et al*., 1998). Moreover, it has a metachromatic characteristics, by intercalation into double stranded or binds electrostatically to single stranded nucleic acids emitting a green fluorescence at 530nm and red fluorescence at 640nm, respectively, under UV light (Kapuscinski *et al*., 1982; Kapuscinski, 1990; Zelenin, 1993). Furthermore, EtBr protocol allows nucleic acid recovery (Sambrook *et al*., 1989) like AO which is also, at the concentration used, would not interfere with nucleic acid recovery and allowing further molecular analysis. It was demonstrated that the luminescence of AO-stained nucleic acid could be completely removed by washing the gels and that nucleic acid can be recovered for reverse transcription, demonstrating the possibility for further molecular studies. AO is also known as supravital dye and is cytotoxic only at high concentrations of about 1.0–10mM (Zelenin, 1993). After intravenous injection of AO (0.1mg/kg) to dogs, it is safe for 1, 3, 7, and 30 days after administration without any toxicity among clinical signs, CBC and serum chemistry values (Maruo *et al*., 2012). Some authors reported usage of AO solution at some human surgical sites without reported toxicity (Coli *et al*., 2006; Kusuzaki *et al*., 2005). It was systemically safe in humans at 500 mg oral dose, except mild gastrointestinal symptoms were observed (Katou, 1970). However, the LD_{50} of intravenous AO was 27.3mg/kg in mice (Satonaka *et al*., 2011), the safe dose was 1 to 10mg/kg intravenous in mice (Hashiguchi *et al*., 2002; Satonaka *et al*., 2010).

Other method for evaluating the DNA abnormalities and genotoxicity screening is micronucleus test which evaluate the abnormal nuclear division leading to structural and numerical chromosomal abnormalities as a result of chromosomal parts occurrence inside the cytoplasm.

However until now, Geimsa and Geimsa / May-Grünwald stains are widely used in micronuclei studies with non epithelial cells originating from a number of organs, such as polychromatic erythrocytes in animal bone marrow (Guzman *et al*., 2004), lymphocytes (Bonassi *et al*., 2003), liver cells (Slamenova *et al*., 2002) and in stable cell lines in vitro studies (Garriott *et al*., 2002). Acridine orange/ethidium bromide (AO/EB) double staining test is fast and statistically significant fluorescence method to determine the (i). Morphological changes in nuclei structures, (ii). Assessing the apoptotic insults, (iii). Easy method to determine the cell viability (Baskic *et al*., 2006). Consequently, AO can be used safely or at least with less hazardous effects as an alternative fluorescent dye for nucleic acids visualization instead of ethidium bromide. It can be also integrated for micronucleus test at the same time of fluorescent viability testing.

MATERIALS AND METHODS

Ethidium bromide [(3,8-diamino-5-ethyl-6-phenyl phenanthridinium bromide)], and acridine orange [3,6-bis(dimethyl) acridinium chloride hemi(zinc chloride salt)] were obtained as a powder (Sigma-Aldrich). All other chemicals were of high analytical grade.

Detection of DNA fragmentation by agarose gel electrophoresis

DNA extraction and detection of fragmentation were carried out according to "salting out extraction method" of Aljanabi and Martinez (1997) with some modifications by Hassab El-Nabi and Elhassaneen (2008). Twenty milligrams of rat's liver tissues were lysed in lysing buffer (10 mM Tris base, 10 mM NaCl, 10 mM Na_2 EDTA, 0.5% SDS, pH 8.3) overnight at 37°C then; 4M NaCl was added to the samples. After centrifugation of the mixture at 10,000 rpm for 10 minutes, the supernatant was transferred to a new tube then DNA was precipitated by 1ml cold isopropanol by centrifugation for 5 min at 12.000 rpm. Pellets were washed with cold 70% ethanol, then they were re-suspended in 10% glycerol in TE buffer (10 mM Tris, 1mM EDTA, pH 8). 10% loading mixture (1X) was added to extracted samples and stored at -20^{o}C until use.

Incubation at 37^{o}C for 30 - 60 minutes with 0.1% RNase was carried out according to the tested samples whether RNA evaluation was needed or not.

Evaluation of nucleic acids fragments

Gels were visualized on UV transilluminator and digitally photographed. The apoptotic bands of DNA fragmentation appeared at 180 bp and its multiples 360, 540 and 720 bp against DNA marker (100–3000 bp). The intensity of DNA fragmentation were measured by (Image J software) as a mean of optical density values.

Induction of apoptosis in rat's liver tissues

The apoptotic fragmentation was induced using post-mortal method according to Hassab El-Nabi and Elhassaneen (2008).

Agarose gel preparation

Gels were prepared using 1.8% normal melting electrophoretic grade agarose in 1X Tris borate EDTA buffer (89 mM Tris, 89 mM boric acid, 2mM EDTA, pH 8.3) at 65 volts until tracking dyes had migrated to the half length of the gel.

Pre-electrophoresis gel staining

Fluorescent nucleic acid dyes either EtBr (5µg/ml) or AO (10µg/ml) were added to the liquid agarose gel at 55^{o}C

before casting in the electrophoresis unit. After gel solidification, nucleic acid samples (with no fluorescent staining) were loaded on the gel and electrophoresed. Representative photographs were captured and evaluated as mentioned previously.

Direct incubation staining of DNA samples

In this case, EtBr (0.25mg/ml) or AO (0.5mg/ml) were incubated directly with DNA samples for 10 minutes then they were loaded on agarose gels containing no fluorescent nucleic acid dye. Electrophoresis and photographing was carried out as mentioned above.

Micronucleus detection by acridine orange/ethidium bromide dual fluorescent staining:

Firstly, human peripheral blood leukocytes were isolated from whole volunteer blood by incubation with four volume folds of erythrocyte lysing buffer (0.015M NH_4C1, 1mM $NaHCO_3$, 0.l mM EDTA). Then, they were centrifuged for 5 minutes at 1000 rpm using cooling centrifuge (Sigma 3K 30, Germany). These steps were repeated until a white pellet appeared (El-Garawani, 2015).

Secondly, nine microliters of peripheral leukocytes suspension were mixed with one microliter of dye mixture (1:1) of acridine orange (50 µg/ml)/ethidium bromide (5 µg/ml) on a clean glass slide, then cells were immediately viewed under fluorescent microscope (Olympus BX 41, Japan) at 40x magnification. About 500 cells were observed and counted in each sample for micronucleus evidence according to the criteria of Tolbert *et al*. (1992) among viable green cells only. Then the representative photos were digitally captured (100 x).

RESULTS

DNA incubation with fluorescent dyes

In this study, AO and EtBr direct incubation with DNA samples revealed more obvious and distinct apoptotic fragmentation rather than the flam shape or necrotic-like pattern of isolated DNA electrophoresis as shown in Figure 1, this was evaluated as optical densities (OD) revealing more OD records in case of EtBr than AO in both liver apoptotic DNA sample and PCR product (Table 1). Moreover, the integration of AO as alternative dye is successfully applied revealing good illumination results of DNA fluorescent staining on agarose gel of the same DNA sample with fixed electrophoresis conditions. The results were obvious either in PCR product electrophoresis on pre-stained gels or with apoptotic DNA sample with direct incubation staining method (Figs. 1-2 and Table 1).

Table 1. The optical densities of rat's liver DNA sample showing the effect of direct incubation with acridine orange and ethidium bromide.

	DNA direct staining	Gel staining	PCR product
EtBr	105.7±5.2	78.6±5.6	89.1±4.9
AO	80.8±6.4	66.9±3.8	74.5±3.5

Data were presented as mean and standard error of three independent measurements.

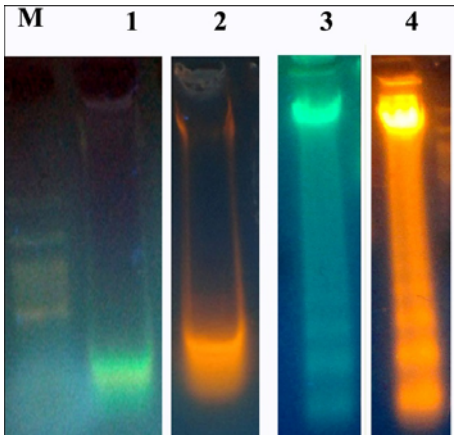

Fig. 1. Representative photomicrograph shows rat's liver DNA sample electrophoresis on 1.8% agarose gel, where, 1: acridine orange pre-stained gel, 2: ethidium bromide pre-stained gel, 3: direct incubation staining with acridine orange on free-stain gel, 4: direct incubation staining with ethidium bromide on free-stain gel and M: DNA marker.

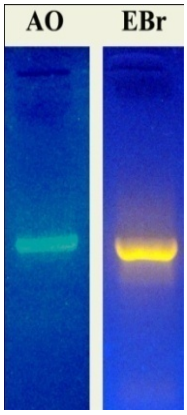

Fig. 2. Representative photomicrograph showing PCR product electrophoresis on 1.8% pre-stained agarose gel showing AO: acridine orange and EBr: ethidium bromide.

Micronucleus detection by acridine orange/ethidium bromide dual fluorescent staining

The obtained results of prephiral blood leucocytes examination for micronucleus occuurance after flourescent staining revealed the obvious distinguished micronucleated viable cells (Fig. 3) with green color while the dead cells with orange to red color were also distinguished.

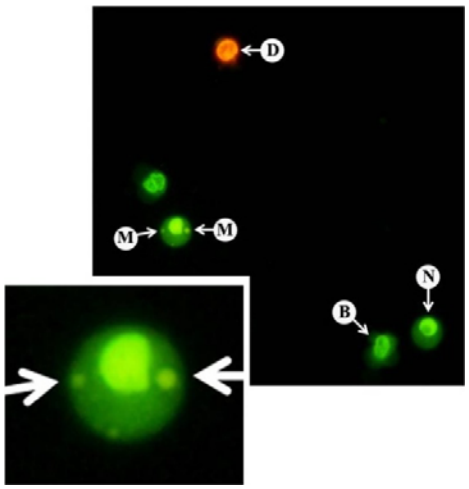

Fig. 3. Representative photomicrograph shows the micronucleated mononuclear human peripheral leucocytes after staining with acridine orange/ ethidium bromide dual fluorescent staining (100x). Where, N: normal viable cell, B: cytoplasmic blebbed cell, M: micronucleated viable cell and D: dead cell.

DISCUSSION

The relative specificity of ethidium bromide (EtBr) for nucleic acid is probably due to the high negative charge of phosphate groups, sugars and hydrogen bonding opportunities in DNA and RNA, and this is also applied for acridine orange (AO) (Bruno *et al*., 1996). However, the interaction of ethidium bromide with nucleic acid is similar to that of acridine orange, except that it does not display metachromatic properties, EtBr can be replaced by AO.

The electrophoretic mobility of DNA molecules depends on its molecular weight. Consequently, DNA-dye adducts formation may affect the DNA migration on agarose gels causing usually shifting or retardation leading to a change in the DNA mobility and more obvious and distinct separation of DNA fragments. This type of interaction ("strong binding") enhances the stability of the double helix against thermal denaturation and the intercalated dye exhibits green luminescence (Kapuscinski and Darzynkiewicz, 1983). This is may be the main core of the obvious and distinct apoptotic fragmentation results after AO and EtBr direct incubation with DNA samples in

this study. Moreover, in line with our hypothesis EtBr and AO are normally used to probe DNA structure in drug–DNA and protein–DNA interactions. They bind DNA and RNA via intercalative mode and slip between adjacent base pairs and cause stretch of double helical structure. The binding of these pigments to double-stranded DNA greatly enhances its fluorescence intensity and lifetime (Nafisi *et al*., 2007). EtBr has the ability to bind with core histones of DNA nucleosome structure (Banerjee *et al*., 2014). All that may be also the core of explanation for the obtained results of direct DNA samples staining method which revealed more obvious apoptotic fragmentation with laddering pattern rather than the flamed smears with ordinary gel staining method. One of other AO advantages is metachromatic properties as it can distinguish between single and double stranded nucleic acids. Interactions of AO with single stranded (ss) nucleic acids or with double strand (ds) nucleic acids at high binding density are less understood. These interactions ("weak" or "external" binding) were originally visualized as formation of dye stacks attached electrostatically to the phosphates of the nucleic acid backbone; the dye-dye interactions in the stacks have been held responsible for the long wavelength (red) luminescence of the AO-ss nucleic acid complexes (Kapuscinski and Darzynkiewicz, 1983). It was further inferred that some conformational rigidity of single-stranded RNA may partially be responsible for the weaker red fluorescence (Ichimura, 1975).

Acridine orange/ethidium bromide dual fluorescent staining method is considered as a rapid, cheap and easy-to-perform assay to investigate the viable and non viable cells (Ciniglia *et al.*, 2010). Moreover, micronucleus test is a well-known test for the genotoxicity screening as a biomarker of structural and numerical chromosomal damage. However until now, most studies have been performed using Geimsa or Geimsa / May-Grünwald, non DNA-specific staining, on smeared cells which consume more time in smears preparations, fixation and staining before visualization. This study provides dual technique to be performed at the same time and preparations using AO as a fluorescent DNA-specific staining to nuclear DNA and micronuclei with green color in parallel to performing viability testing. AO staining proved at least more equally observation without staining artifacts and with less time consuming.

CONCLUSION

It was found that although acridine orange staining is less sensitive method than ethidium bromide, its use is safer, and cost efficient. It allows metachromatic properties and nucleic acid can be recovered for further molecular analysis. Acridine orange also can be used for increased sensitivity of apoptosis detection using direct incubation method and integrated for micronucleus technique.

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