ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (3): 514-522, 2008
ISSN 1682-296X
© 2008 Asian Network for Scientific Information

Detection of DNA Damage, Molecular Apoptosis and Production of Home-Made Ladder by Using Simple Techniques

[1]S.E. Hassab El-Nabi and [2]Y.A. Elhassaneen
[1]Department of Zoology, Faculty of Science,
[2]Department of Nutrition and Food Science, Faculty of Home Economics,
Minufiya University, Shebin El-Kom, Egypt

Abstract: Simple, sensitive and rapid techniques were modified for detection of total genomic damage of DNA (TGD-DNA), Electrophoretic pattern of RNA and molecular apoptosis. The techniques were applied in both cell culture and solid tissues. In cell culture, the cells were lysated directly in the gel wells and mixed with loading buffer. In solid tissues, 5-10 mg were squeezed and lysated in ependorf tubes, then 1 mg of lysated tissues was loaded in the gel wells and mixed with loading buffer. This technique elaborated the DNA damage as smear or apoptotic bands. Detection of molecular apoptosis by gel electrophoresis was modified in our laboratory, where, salt out extraction method of DNA was applied using small amount of tissues (5-10 mg), squeezed by blue tips and lysated quickly by lysing buffer. Mixture of loading mix was developed which has a great effect on the stability and resolution of apoptotic bands. The developed technique is very simple, fast and economic to prepare DNA apoptotic ladder from liver, spleen and human leukocytes. Induction of apoptosis was done chemically by methotraxate in liver, spleen and thymus of rats, or naturally by postmortem changes in liver of rat and storage blood of human. Some factors were studied for stability and resolution of apoptotic ladder such concentrations of SDS in lysing buffer, effects of temperatures, glycerol and our loading mix. Concentration of SDS has no effect on the intensity of apoptotic bands in extracted DNA, while in cell or tissue lysate, the apoptotic bands appeared sharp at low concentrations of SDS (0.5-1%). After preparation of DNA and addition of TE buffer the best temperature that keeps on the stability of DNA was 37°C. Our loading mix and glycerol protects apoptotic bands from degradation at room temperature or -20°C. The stability of home made apoptotic ladder was stable for 6 month or more at room temperature

Key words: DNA damage, molecular apoptosis, home-made ladder, postmortem

INTRODUCTION

DNA technology is one of the most important techniques in twenty-one century. Most of diseases are accompanied with DNA damage or disturbance in gene expression (Elizur *et al*., 2006; Ricci *et al*., 2007). Techniques which permit the detection of DNA damage are useful in studies of mutagenicity and carcinogenicity of environmental pollution (Elhassaneen, 1996; Hassab El-Nabi *et al*., 2001; Nassar *et al*., 2003; Li *et al*., 2007). Double strand breakage (DSB) and single strand breakage (SSB) are the main types of DNA damage. Several methods are employed to measure DSB. Amongst of these methods, pulsed field gel electrophoresis (PFGE) was occupied the central position (Schwartz and Cantor, 1984). But, factors including time-consuming (2 days), fine chemicals and equipments required made this work is not easy task. Recently, several protocols were developed in our laboratory to modify this method through direct lysing of the cells in the sample wells of agarose electrophoresis gel (Hassab El-Nabi, 2000) or squeezing and lysate of 5 mg of solid tissue in ependorf tube and 1 mg of lysate tissue loaded to the wells. The new protocols saved the time to 2 h, efforts, equipment's and chemicals required etc. Our previous studies indicated that these modified techniques were used successively to evaluate the DNA damage and the intensity of total RNA in many toxicological and environmental applications (Hassab El-Nabi and Hagras, 2001; El-Beshlawy *et al*., 2003).

Apoptosis is one of the most interesting biological events which has received a great deal of attention for its role in regulating morphogenesis during embryonic stages of development and cellular turnover during post-natal life

Corresponding Author: Prof. Dr. Yousif Abd El-Aziz Elhassaneen, Department of Nutrition and Food Science, Faculty of Home Economics, Minufiya University, Shebin El-Kom, Egypt
Tel: (++) 2 010 502 3515 Fax: (++) 2 048 222 7638

(Cohen and Duke, 1992; Raff, 1992; Williams and Smith, 1993). It has been thus associated with tissue homeostasis and consequently involved in the pathogenesis of a large number of diseases (Miscia *et al.*, 1997). Several techniques were developed for detection of apoptosis. For example, molecular apoptosis detected by gel electrophoresis is used to demonstrate the ladder pattern of DNA (a hallmark of apoptosis) which is generated by endonucleolytic cleavage of genomic DNA into nucleosomal size DNA of approximately 180 bp long (monomers) or oligonucleotides which are multiples of 180 bp (Wyllie, 1995). Many of set backs were coming with this methods of apoptosis detection such the complication of nucleic acid extraction protocol applied, not easy to get the DNA ladder pattern required and its high price and others made this technique not popular.

With the above in mind, we reported here a simple and sensitive gel electrophoresis detection of DNA damage and molecular apoptosis in both cell cultures and solid tissues. In a very important application, this technique has been used to explain the postulated mechanism of apoptosis process in tissues at postmortem intervals. Additionally, through induction of apoptosis chemically or naturally by postmortem changes or storage of blood, this investigation enables a production of home-made DNA ladder in a trail to solve one of the most important problems in pertaining to this kind of techniques.

MATERIALS AND METHODS

Experimental models

Animals: Male albino rats of Sprague Dawley, weighing 140±27 g (males sex) were obtained from Helwan Research Station, Vaccines Association, Ministry of Health and Populations, Cairo, Egypt were housed in an environmentally controlled animal facility operating on a 12 h dark/light cycle at 24-26°C. They were acclimated for 1 week before experimentation. Toad, lizard, pigeon and rabbit were obtained by special arrangements with the Minufiya University suppliers, Sebin El-Kom, Egypt.

Human blood: Blood samples required for leucocytes cultures were withdrawn from volunteers recruited from the Minufiya University Community.

Apoptosis induction: Apoptosis in liver, spleen and the thymus of male albino rats were detected after treatment with methotrexate (Orion Pharma, vial 50 mg mL^{-2}). Apoptosis also detected in rat liver at different postmortem intervals. Also apoptosis was detected in leukocytes of stored blood. For studying the effect of methotrexate on DNA, a group of 6 rats were assigned to three subgroups each of 2 rats. Subgroup (a): Animals of these subgroup were considered as a control and injected intraperitonealy (i.p.) with 0.1 mL distilled water. Subgroup (b): Two animals were injected i.p. with one dose of methotrexate equivalent to 90 mg kg^{-1} and sacrificed after 24 h. Subgroup (c): Two animals were injected i.p. with one dose of methotrexate 150 mg kg^{-1} body weight and sacrificed after 24 h. For study the effect of postmortem interval on DNA, Two rats were sacrificed and liver was excised and kept at 37°C. 0.01 g of liver were squeezed at intervals of 0. 0, 3, 6, 9, 18 and 24 h and was lysed by lysing buffer. For study the apoptotic activity of DNA in stored human blood, 10 mL blood from healthy non-smoker were classified to two parts each is 5 mL, human leukocytes isolated from one part (fresh blood) and second part after storage in refrigerator for 5 days. Human leukocytes were lysed by lysing buffer and incubated at 37°C overnight.

Methods: Total genomic damage of DNA in leukocytes were detected according to Wlodek *et al.* (1991) and modification by Hassab El-Nabi (2000). Electrophoretic pattern of nucleic acids for solid tissue were demonstrated according to Hassab El-Nabi (2004). Human leukocytes (2×10^4) were loaded in well. Eighteen micro-liter of lysing buffer (50 mM NaCl, 1 mM Na_2EDTA, 0.5% SDS, pH 8.3) was added on the cells for 30 min. Five microlitres from 6X loading buffer was added on the lysis cells. DNA damage or electrophoretic pattern of nucleic acids of solid tissue was detected in lysate tissue (Hassab El-Nabi *et al.*, 2001). Where, a piece of 10 mg of each of the liver and spleen was squeezed by blue tips and lysed with 200 μL lysing buffer. After 1 h, 20 μL was loaded on gel to detect DNA damage. Electrophoresis was performed for 2 h at 50 volt using 1 X TBE buffer as running buffer. Gel was photographed using a polaroid camera while the DNA was visualized using a 312 nm UV light under a transilluminator.

Nucleic acids extraction and detection of apoptosis in solid tissue and human leukocytes were done according to salting out extraction method of Aljanabi and Martinez (1997) and modification introduced by Hassab El-Nabi (2004). Protein was precipitated by saturated sol of NaCl (5 M). The method of extraction is summarized as follows: In case of solid tissue, 10 mg of liver was squeezed and lysed with 600 μL of lysing buffer. Also leucocytes (3×10^4 cells) was isolated from blood according to Papaconstantinou *et al.* (2001) and lysed with 600 μL lysing buffer (50 mM NaCl, 1 mM Na_2

EDTA, 0.5% SDS, pH 8.3) and gently shacked. The mixture was incubated overnight at 37°C then, 200 μL of saturated NaCl was added to the samples, shacked gently and centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to new eppendorf tubes and then DNA precipitated by 600 μL cold isopropanol. The mix was inverted several times till fine fibers appear and then centrifuged for 5 min at 12,000 rpm. The supernatant is removed and the pellets were washed with 500 μL 70% ethyl alcohol, centrifuged at 12,000 rpm for 5 min. After centrifugation, the alcohol was decanted or tipped out and the tubes blotted on Whatman paper or clean tissue, till the pellets appeared to be dry. The pellets were resuspended in 50 μL or appropriate volume of TE buffer (10 mM tris, 1 mM EDTA, pH 8) supplemented with 5% glycerol. The resuspended DNA was incubated for 30-60 min with loading mix (RNAse + loading buffer) and then 2 μg of DNA was loaded directly into the gel-wells. The concentration of DNA was measured at A_{260}, the yield of DNA in liver was 550 ng mg^{-1} fresh weight.

Data analysis: Electrophoretic pattern of nucleic acids of leukocytes and liver appeared as three main bands in gel. RNA area located at up 150 bp, nucleoprotein area located at 300-350 bp and DNA area located near the wells of gel to nucleoprotein area. In case of apoptosis, apoptotic DNA bands appeared at 180-200 bp and its multiply. The DNA and RNA have orange color with ethidium bromide, while, nucleoprotein has a purple color. When the gel exposed to UV by UV-transilluminator for 5 min, the color of nucleoprotein change from purple to white. After staining the gel with commassi blue the DNA and RNA not stained, while, the nucleoprotein stained darkly with blue color.

Apoptotic pattern of DNA appeared in gel as sharp and thick bands, the length of each band about 20 bp. The bands located at 180-200 bp (one edge located at 200 bp and the opposite edge located at 180 bp) and its multiply.

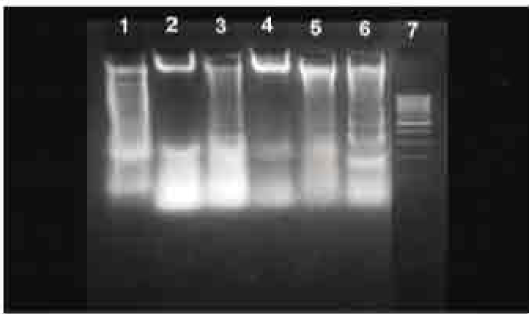

Fig. 1: Effect of methotrexate on electrophoretic pattern of nucleic acids in liver and spleen of rat. Lane 1, untreated liver cells; lane 2, liver treated with 90 mg kg^{-1} methotrexate; lane 3, liver treated with 150 mg kg^{-1} methotrexate; lane 4, untreated spleen cells; lane 5, spleen treated with 90 mg kg^{-1} methotrexate; lane 6, spleen treated with 150 mg kg^{-1} methotrexate; lane 7, standard markers (1 Kb ladder)

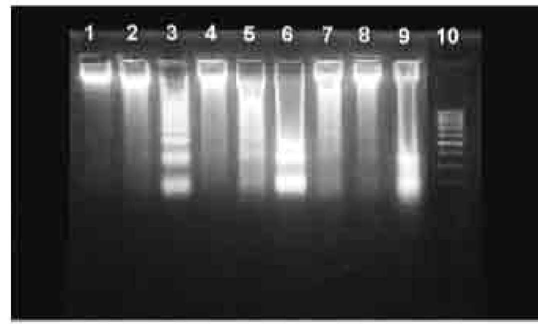

Fig. 2: Effect of methotrexate on DNA of liver, spleen and thymus of rat. Lane 1, untreated liver cells; lane 2, liver treated with 90 mg kg^{-1} methotrexate; lane 3, liver treated with 150 mg kg^{-1} methotrexate; lane 4, untreated spleen cells; lane 5, spleen treated with 90 mg kg^{-1} methotrexate; lane 6, spleen treated with 150 mg kg^{-1} methotrexate; lane 7, untreated thymus cells; lane 8, thymus treated with 90 mg kg^{-1} methotrexate; lane 9, thymus treated with 150 mg kg^{-1} methotrexate; lane 10, standard markers (1 Kb ladder)

RESULTS

Effect of methotrexate on electrophoretic pattern of nucleic acids in liver and spleen of rat: Methotrexate increased the intensity of RNA especially at 25 mg kg^{-1}. Also it induced DNA damage which appeared as a apoptotic bands and located at 180 bp with its multiply as shown in Fig. 1.

Effect of methotrexate on DNA of liver, spleen and thymus: As shown in Fig. 2, methotrexate induced apoptosis in liver, spleen and thymus of rat after 24 h of treatment. The intensity of apoptotic bands increased with dose dependent-manner. At 25 mg kg^{-1} of methotrexate, liver give good profile of 200 bp DNA ladder while, spleen give three sharp bands located at 200, 400 and 600 bp and thymus gives only two main sharp bands located at 200 and 400 bp. The intensity of apoptotic bands revealed the level of apoptosis induction. Methotrexate induced more apoptosis (DNA fragmentation) in thymus than spleen and in spleen than liver as shown in Fig. 2.

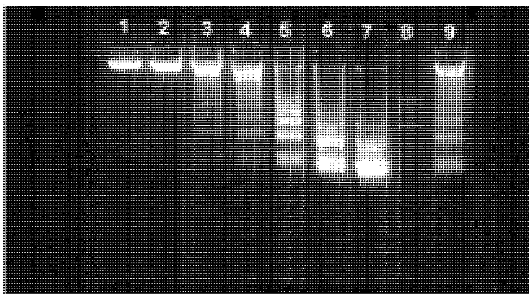

Fig. 3: Effect of postmortem on DNA of rat liver. Lane 1, Zero time liver cells; lane 2, 3 h of postmortem; lane 3, 6 h; lane 4, 9 h; lane 5, 12 h; lane 6, 18 h; lane 7, 24 h; lane 8, standard markers (1 Kb ladder); lane 9, Home-made ladder from leucocytes

Effect of postmortem on DNA of rat liver: DNA profile was demonstrated at 0.0, 3, 6, 9, 12, 18 and 24 h of postmortem. At zero time DNA is intact and no bands detected. After 3 h DNA is still intact but faint apoptotic bands begin to appear. At 6 and 9 h of postmortem, DNA gives a good profile of 200 bp DNA ladder. At 12, 18 and 24 h of postmortem, the numbers of sharp apoptotic bands were 5, 4 and 3, respectively. The apoptotic bands located at 200, 400, 600, 800 and 1000 bp as shown in Fig. 3.

Effect of different concentrations of SDS in lysing buffer on the intensity of apoptotic bands in rat liver induced by postmortem: Different concentrations of SDS 0.5, 1, 2, 3 and 4% in lysing buffer were applied at liver incubated at 37°C for 0 and 18 h. At zero time of incubation, DNA is intact at 0.5, 1 and 2% of SDS, while at 3 and 4% of SDS, fine apoptotic bands were appeared. At 24 h of postmortem, liver give a good profile of 200 bp DNA ladder, the intensity of apoptotic bands has a negative correlation with the concentrations of SDS. The resolutions of apoptotic bands were sharp at 0.5, 1 and 2% than 3 and 4% of SDS as shown in Fig. 4.

Effect of glycerol and modified loading mix on the stability of apoptotic bands of prepared DNA of rat liver at different temperature: Immediately, after preparation of DNA of liver at 18 h of postmortem, DNA was distributed in three sets of eppendorf tubes, each set consists of three eppendorf. The 1st tube has a DNA in TE buffer only, the 2nd one tube has a DNA in TE buffer +10% glycerol, the 3rd tube has a DNA in TE buffer + modified loading mix. The 1st set of tubes was incubated overnight at 37°C, the 2nd set was kept at room temperature and the 3rd set was kept at -20°C overnight. As shown in Fig. 5, the stability of apoptotic bands were demonstrated at

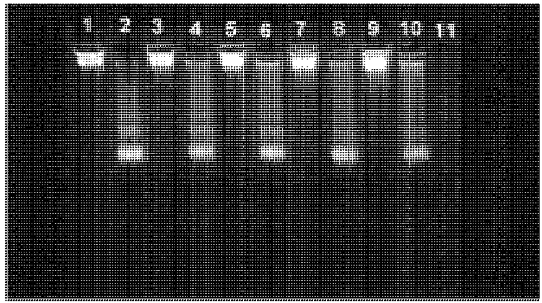

Fig. 4: Effect of different concentrations of SDS in lysing buffer on the intensity of apoptotic bands in rat liver induced by postmortem. Lane 1, Zero time liver cells (0.5% SDS); lane 2, 18 h of postmortem (0.5% SDS); lane 3, Zero time liver cells (1.0% SDS); lane 4, 18 h of postmortem (1.0 % SDS); lane 5, Zero time liver cells (2.0% SDS); lane 6, 18 h of postmortem (2% SDS); lane 7, Zero time liver cells (3% SDS); lane 8, 18 h of postmortem (3% SDS); lane 9, Zero time liver cells (4% SDS); lane 10, 18 h of postmortem (4% SDS); lane 11, standard markers (1 Kb ladder)

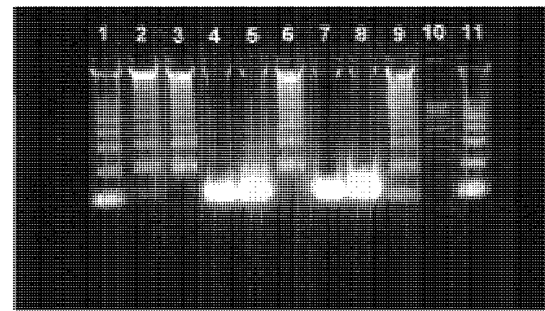

Fig. 5: Effect of glycerol and modified loading mix on the stability of apoptotic bands of prepared DNA of rat liver at different temperature. Lane 1, DNA in TE buffer (37°C); lane 2, DNA in TE buffer + 10% glycerol (37°C); lane 3, DNA in TE buffer + modified loading mix (37°C); lane 4, DNA in TE buffer (room T); lane 5, DNA in TE buffer + 10% glycerol (room T); lane 6, DNA in TE buffer + modified loading mix (room T); lane 7, DNA in TE buffer (-20°C overnight); lane 8, DNA in TE buffer + 10% glycerol (-20°C overnight); lane 9, DNA in TE buffer + modified loading mix (-20°C overnight); lane 10, standard markers (1 Kb ladder); lane 11, Home-made ladder from spleen cells

37°C, while the apoptotic bands start to disappeared at room temperature and disappeared completely at -20°C. Addition of glycerol to DNA kept the stability of

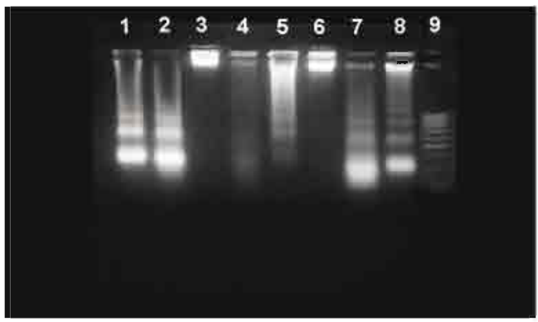

Fig. 6: Induction of apoptosis in liver of different classes of animal kingdom by postmortem. Lane 1, Zero time liver cells of toad; lane 2, 18 h of postmortem; lane 3, Zero time liver cells of lizard; lane 4, 18 h of postmortem; lane 5, Zero time liver cells of pigeon; lane 6, 18 h of postmortem; lane 7, Zero time liver cells of rabbit; lane 8, 18 h of postmortem; lane 9, standard markers (1 Kb ladder)

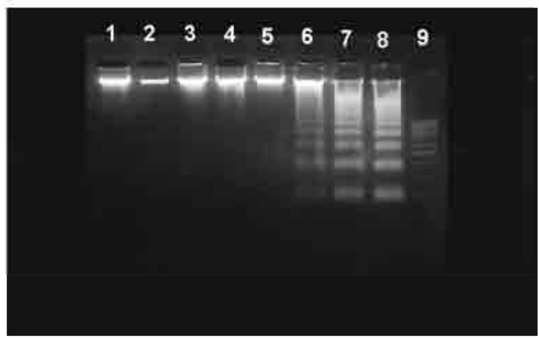

Fig. 7: Effect of storage on DNA of blood leukocytes. Lanes 1-4, fresh human blood; lanes 5-8, blood stored for one week at 4°C; lane 9, standard markers (1 Kb ladder)

apoptotic bands at 37°C and at room temperature. Our modified loading mix, kept on the stability of apoptotic bands at 37°C room temperature and -20°C giving a good profile of 200 bp DNA ladder.

Induction of apoptosis in different classes of animal kingdom: Apoptosis was inducted in liver of amphibian (toad), reptiles (lizard), birds (pigeon) and mammals (rabbit) by postmortem. Apoptotic bands were sharp in toad, pigeon and rabbit. The liver of rabbit gives a good profile of 200 bp DNA ladder as shown in Fig. 6.

Effect of storage on DNA of blood leukocytes: Intact and apoptotic DNA ladder were detected after storage of blood at zero and one week at 4°C, respectively. Blood storage gives a good pattern of 200 bp DNA ladder as shown in Fig. 7.

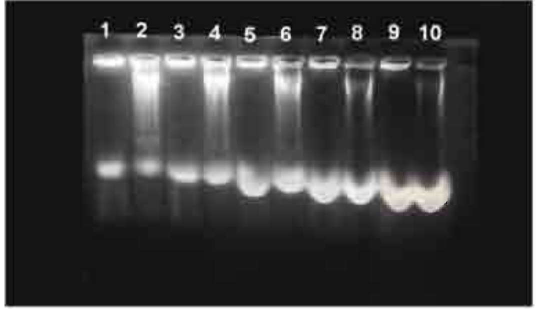

Fig. 8: Effect of SDS concentration on the total genomic damage of DNA of human blood leucocytes, fresh and stored (3 days at 4°C) samples. Lane 1, fresh blood (0.5% SDS); lane 2, stored blood (0.5% SDS); lane 3, fresh blood (1% SDS); lane 4, stored blood (1% SDS); lane 5, fresh blood (2% SDS); lane 6, stored blood (2% SDS); lane 7, fresh blood; lane 8, stored blood (3% SDS); lane 9, fresh blood (4% SDS); lane 10, stored blood (4% SDS); lane 11, standard markers (1 Kb ladder)

Effect of SDS concentration on total genomic damage of DNA: Different concentrations of SDS 0.5, 1, 2, 3 and 4% in lysing buffer was applied for lysing leukocytes separated from fresh and stored blood for 3 days at 4°C. In fresh blood no DNA damage was detected, on the other hand, observable DNA damage and faint apoptotic bands were detected in stored blood. The intensity of RNA in fresh blood was more than in stored blood. The size of nucleoprotein area was increased with increasing the concentrations of SDS, while, the intensity of apoptotic bands decreased with increasing the concentrations of SDS. The best concentrations of SDS for detecting total DNA damage in leukocytes was 0.5 and 1% as shown in Fig. 8.

Effect of SDS concentration on protein pattern of human blood leukocytes: The gel with different concentrations of SDS was stained with commassi blue to show the profile of protein at different concentrations as shown in Fig. 9. The intensity of nucleoprotein increased with increasing the dose of SDS while, the intensity of protein in the area of DNA decreased with increasing of the concentrations of SDS.

Effect of SDS concentrations on the intensity of the apoptotic bands in human blood leukocytes: Different concentrations of SDS 0.5, 1, 2, 3 and 4% were used in lysing the human leukocytes of fresh and stored blood for 3 days at 4°C. Apoptotic 200 bp DNA ladder was produced from stored blood. The intensity of apoptotic

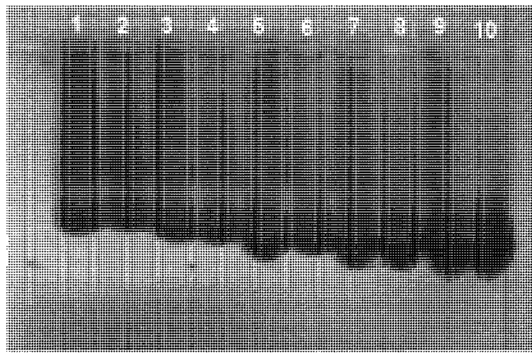

Fig. 9: Effect of SDS concentration on protein pattern of human blood leucocytes, fresh and stored (3 days at 4°C) samples. Lane 1, fresh blood (0.5% SDS); lane 2, stored blood (0.5% SDS); lane 3, fresh blood (1% SDS); lane 4, stored blood (1% SDS); lane 5, fresh blood (2% SDS); lane 6, stored blood (2% SDS); lane 7, fresh blood; lane 8, stored blood (3% SDS); lane 9, fresh blood (4% SDS); lane 10, stored blood (4% SDS); lane 11, standard markers (1 Kb ladder)

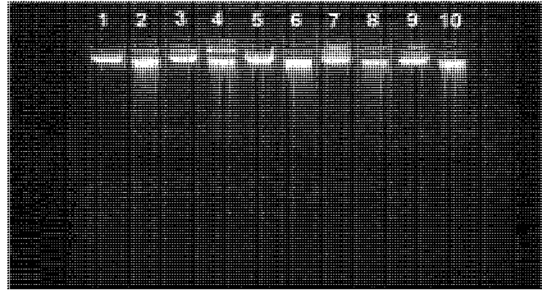

Fig. 10: Effect of SDS concentrations in lysing buffer on the intensity of the apoptotic bands in human leukocytes of fresh and stored (3 days at 4°C) blood samples. Lane 1, fresh blood (0.5% SDS); lane 2, stored blood (0.5% SDS); lane 3, fresh blood (1% SDS); lane 4, stored blood (1% SDS); lane 5, fresh blood (2% SDS); lane 6, stored blood (2% SDS); lane 7, fresh blood; lane 8, stored blood (3% SDS); lane 9, fresh blood (4% SDS); lane 10, stored blood (4% SDS); lane 11, standard markers (1 Kb ladder)

bands at concentrations of 0.5 and 1% of SDS were sharp and more than that obtained by concentrations of 2, 3 and 4% of SDS as shown in Fig. 10.

DISCUSSION

The present study offer some modified molecular techniques that could detect DNA damage, electrophoretic pattern of nucleic acids and molecular detection of apoptosis in human leukocytes culture and in solid tissues. These modified techniques are very simples, rapid, universal applicable, not required expensive and environmental hazardous reagents and equipment's. In the present study, methotrexate (MTX) induced apoptosis (programmed DNA fragmentation) in liver, spleen and thymus gland. It has been suggested that MTX binds to dihydrofolate reductase (DHFR) and completely inhibits the activity of this enzyme. The continuous inhibition of DHFR activity might cause an imbalance in the deoxyribonucleotide triphosphate (dNTP) pool due to the storage of thymidylate and purine nucleotides and as a consequence lead to DNA lesions (Li and Kaminskas, 1984). Previous studies reported that methotrexate induced apoptosis in cultured bovine pulmonary artery endothelial cells, in rat intestinal epithelial cells, U937 cell line and hepatocytes of rat (Merkle *et al*., 2000; Xiao *et al*., 2002; Kobayashi *et al*., 2002). For explaining the mechanism of apoptosis induced by methotrexate, Kobayashi *et al*. (2002) suggested that the inhibition of JNK-AP1, c-Jun N-terminal kinase-activator protein 1, pathway and concurrent up-regulation of 53 and p21 were involved in hepatocyte apoptosis. Methotrexate induces gut mucosal apoptosis *in vivo*, however, little is known about the molecular mechanism involved. Methotrexate induced apoptosis and decreased cell number in rat intestinal epithelial cells (RIE-1). DNA fragmentation was preceded by the sequential activation of caspase 9, 2 and 3, whereas, caspase 1 and 8 remained inactive (Papaconstantinou *et al*., 2001).

In the present study apoptosis was detected in liver of rat after death. The level of DNA degradation or apoptosis has a direct correlation with postmortem intervals. The effect of postmortem intervals on DNA damage or apoptosis were studied in different organs of rat, spleenic tissue and dental pulp and hepatic tissue (Kanetake *et al*., 1998; Johnson and Ferris, 2002; Di Nunno *et al*., 2002; Boy *et al*., 2003). The mechanism of apoptosis that resulted from postmortem may be due to autolysis and the internal nucleases activity (Kanetake *et al*., 1998; Boy *et al*., 2003). DNA fragmentation (apoptosis) *in situ* in Flash-frozen human postmortem midbrain has been reported by Kingsbury *et al*. (1998). This process was influenced by antemortem hypoxia and that apoptosis was seen in the postmortem brain may parallel to those observed in experimental ischemia in the animal brain.

The present study revealed apoptosis in blood stored at 4°C for 1 week. As well, apoptosis was detected in stored granulocytes, lymphocytes, whole blood and peripheral blood mononuclear cells (Frabetti *et al*., 1998; Fowke *et al*., 2000). Platelets possess some of the

machinery required for apoptotic cell death in stored blood. Li *et al.* (2000) reported that platelets display biochemical sign of apoptosis after 5 days of storage under standard blood banking. Platelets contain many of the components of the apoptotic mechanism and show activation of caspase 3 and consequent cleavage of gelsolin during blood storage.

In this study the addition of glycerol to immediately prepared DNA keeps the stability of apoptotic bands at room temperature. Hu and O'Shaugnssy (2001) reported that the addition of glycerol to polyacrylamide gel electrophoresis improved the resolution of electrophoretic bands of DNA. Ohnishi *et al.* (2000) reported that glycerol able to restores p53-dependent radiosensitivity of human hand and neck cancer cells baring mutant p53. They suggested that glycerol is effective in inducing conformational changes of p53 and restoring normal function to mutant p53. Nitroglycerin has cytoprotective effects in ischmia-reperfusion induced lung injury (Kawashima *et al.*, 2000). It has been noted that addition of glycerol to the gel containing tris-borate buffer enhances the sensitivity of single-strand conformation polymorphism analysis of PCR products. The effect of glycerol is caused by the reduction of pH of the buffer through the reaction of glycerol and borate ion (Kukita *et al.*, 1997).

Mitochondrial and lysosomal pathways are the main ways for induction of apoptosis. Recent studies suggested that the lysosomal protease cathepsine D, when released from lysosomes to cytosol, can initiate apoptosis. For example, Boya *et al.* (2003) reported that lysosomal membrane permeabilization (LMP) induces cell death in a mitochondrion-dependent fashion. Ciprofloxacin (CPX) and norfloxacin (NFX) are quinolone antibiotics, they induce LMP as detected by the release of cathepsins from lysosomes. LMP triggers mitochondrial membrane permeabilization (MMP) as detected by the release of cytochrome C (Boya *et al.*, 2003). In another study, Milosevic *et al.* (2003) reported that apoptosis induced by DNA-damaging agents or radiation mainly proceeds through death receptor-independent caspase activation. The release of mitochondrial apoptotic proteins, such as cytochrome c, into the cytoplasm leads to activation of caspase-9 which is a key event in apoptosis process. Boya *et al.* (2003) reported that hydroxychloroquine (HCQ) is a lysosomotropic amine with cytotoxic properties. This compound induces signs of lysosomal membrane permeabilization (LMP), such as the decrease in the lysosomal pH gradient and the release of cathepsin B from the lysosomal lumen, followed by signs of apoptosis including caspase activation, phosphatidylserine exposure and chromatin condensation with DNA loss. A caspase-activated DNase (CAD) has been reported by Enari *et al.* (1998). CAD is a 40 kDa that induces internucleosomal DNA cleavage in isolated nuclei in the presence of Mg^{2+}. AN34, a nuclease enzyme induced DNA breaks terminate with 3-OH consistent with characteristic products of apoptotic chromatin fragmentation. Interestingly, the action of AN34 as a nuclease enzyme is controlled by caspase that is responsible for the induction of apoptosis (Yoshida *et al.*, 1998). In additional, Kagedal *et al.* (2001) reported that sphingosine which is a detergent induced lysosomal rupture and apoptosis at moderate concentrations in Jurkat and j774 cells. High concentrations of sphingosine rapidly caused extensive lysosomal rupture and ensuing necrosis without antecedent apoptosis or caspase activation. They conclude that lysosomal cathepsin D and one or more cysteine proteases, such as cathepsines B or L, are important mediators of sphingosine-induced apoptosis, working upstream of the caspase cascade and mitochondrial membrane-potential changes (Kagedal *et al.*, 2001).

For explaining the apoptosis in tissues at postmorem intervals, accumulating information now suggests that lysosomal cathepsins are pivotally involved in apoptosis process. The role of lysosomes and lysosomal enzymes in initiation and execution of the apoptotic program has become clear in several models, to the point that the existence of a lysosomal pathway of apoptosis is now generally accepted. This pathway of apoptosis can be activated by death receptors, lipid mediators, photodamage. Lysosomal proteases can be released from the lysosomes into the cytosol, where they contribute to the apoptotic cascade upstream of mitochondria (Guicciardi *et al.*, 2004).

In conclusion, the present research offered simple modified techniques that detect DNA damage, electrophoretic pattern of RNA and molecular apoptosis in both cell cultures and solid tissue. All of the conditions required for optimization of the technique have been studied. Also, the modified technique used successfully in many molecular biology applications including explanation of the postulated mechanism of apoptosis process in tissues at postmortem intervals and production of home-made DNA ladder with sufficient amount through induction of apoptosis by chemically or naturally methods.

REFERENCES

Aljanabi, S.M. and L. Martinez, 1997. Universal and rapid salt extraction of high quality genomic DNA for PCR based technique. Nucleic Acid. Res., 25: 4692-4693.

Boy, S.C., H. Bernitz and W.F. Van Heerden, 2003. Flow cytometric evaluation of postmortem pulp DNA degradation. Am. J. Forensic Med. Pathol., 24: 123-127.

Boya B., R.A. Gonzalez-Polo, D. Poncet, K. Andreau, H. L. Vieira, T. Roumier, J.L. Perfettini and G. Kroemer, 2003. Mitochondrial membrane permeabilization is a criticalstep of lysosome-initiated apoptosis induced by hydroxychloroquine. Oncogen, 22: 3927-3936.

Cohen, J.J. and R.C. Duke, 1992. Apoptosis and programmed cell death in immunity. Annu. Rev. Immunol., 10: 267-293.

Collins, M.K. and A.L. Rivas, 1993. The control of apoptosis in mammalian cells. Trends Biochem. Sci., 18: 307-309.

Di Nunno, N., F. Costantinides, S.J. Cina, C. Rizzared, C. Di Nunno and M. Melato, 2002. What is the bst sample for determining the early postmortem period by on the spot flow cytometry analysis? Am. J. Forensic Med. Pathol., 23: 173-180.

El-Beshlawy, N., S.E. Hassab El-Nabi, M. Abdalla, A.K. Kamel, H.M. Bhr and N.A. El-Mahdi, 2003. Assessment of genome damage in a population of Egyptian workers occupationally exposed to cotton dusts. AinShams J. Forensic Med. Clin. Toxicol., 1: 1-24.

Elhassaneen, Y.A., 1996. Biochemical and technological on pollution of fish with pesticides and polycyclic aromatic hydrocarbon. Ph.D Thesis, Mansoura University, Mansoura, Egypt.

Elizur, T., R .Yosef, D. Elinger, A. Vexler, M. Krupsky, A. Berrebi, A. Shani, E. Schechtman, L. Freedman and Z. Livneh, 2006. Reduced repair of the oxidative 8-Oxoguanine DNA damage and risk of head and neck cancer. Cancer Res., 66: 11683-11689.

Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu and S. Nagata, 1998. A caspase-activated DNase that degrades DNA during apoptosis and its inhibitor ICAD. Nature, 391: 43-50.

Fowke, K.R., J. Behnke, C. Hanson, K. Shea and L.M. Cosntino, 2000. Apoptosis: A method for evaluating the cryopreservation of whole blood and peripheral blood mononuclear cells. J. Immunol. Methods, 244: 139-144.

Frabetti, F., D. Musiani, M. Marini, C. Fanelli, S. Coppola, L. Ghibelli, P.L.M. Tazzari, A. Bontadini, C. Tassi and R. Conte, 1998. White cell apoptosis in packed red cells. Transfusion, 38: 1082-1089.

Guicciardi, M.E., M. Leist and G.L. Gores, 2004. Lysosomes in cell death. Oncogene, 16: 2881-2890.

Hassab El-Nabi, S.E., 2000. Rapid and simple technique for detection of total genomic damage (TGD) of DNA in human lymphocytes and its application on some mutagenic agents. J. Union Arab Biol., 14A: 371-388.

Hassab El-Nab, S.E. and G.M. Hagras, 2001. Praziquantel (distocide) and *Commiphora molmol* extract (merazid) on human lymphocytes culture. Zagazig Univ. Med. J., 5: 2255-2276.

Hassab Elnabi, S.E., A.H. Mohamed and G.Y. Osman, 2001. Estimation of RNA electrophoretic pattern as an indicator of pollution in *Biomphalaria alexandrina* snails treated with certain plant growth regulators, a herbicide and lead acetate. J. Union Arab Biol., 15A: 467-486.

Hassab El-Nabi, S.E., 2004. Molecular and cytogenetic studies on the antimutagenic potential of eugenol in human lymphocytes culture treated with depakine and apetryl drugs. J. Egypt. Ger. Soc. Zool., 43C: 171-196.

Hu, C.T. and K.M. O'Shaughnessy, 2001. Glycerol-enhanced mini-polyacrylamide gel electrophoresis for separation of differentially expressed DNA fragments in Cdna representational difference analysis. Electrophoresis, 22: 1063-1068.

Johnson, L.A. and J.A. Ferris, 2002. Analysis of postmortem DNA degradation by single-cell gel electrophoresis. Forensic Sci. Int., 126: 43-47.

Kagedal, K., M. Zhao, I. Svensson and U.T. Brunk, 2001. Sphingosine-induced apoptosis is dependent on lysosomal proteases. Biochem. J., 359: 335-343.

Kanetake, J., M. Nata, N. Adachi, M. Hashiyada, G. Ji and K. Sagisaka, 1998. Effect of postmortem change on detection of apoptosis in rats. Nippon Hoigaku Zasshi., 52: 144-148.

Kawashima, M., T. Bando, T. Nakamura, N. Isowa, M. Liu, S. Toyokuni, S. Hitomi and H. Wada, 2000. Cytoprotective effects of nitroglycerin in ischemia-reperfusion-induced lung injury. Am. J. Respir. Crit. Care Med., 161: 935-943.

Kingsbury, A.E., C.D. Mardsen and O.J. Foster, 1998. DNA fragmentation in human substantia nigre: Apoptosis or perimortem effect? Mov. Disord., 13: 887-884.

Kobayashi, K., C. Terada and I. Tsukamoto, 2002. Methotrexate-induced apoptosis in hepatocytes after partial hepatectomy. Eur. J. Pharmacol., 438: 19-24.

Kukita, Y., T. Tahira, S.S. Sommer and K. Hayashi, 1997. SSCP analysis of long DNA fragments in low pH gel. Hum. Mutat., 10: 400-407.

Li, J. and E. Kaminskas, 1984. Accumulation of DNA strand breaks and methotrexate cytotoxicity. Proc. Natl. Acad. Sci. USA., 81: 5694-5698.

Li, J., Xia, Y., A.M. Bertino, J.P. Coburn and D.L. Kuter, 2000. The mechanism of apoptosis in human platelets during storage. Transfusion, 40: 1320-1329.

Li, N., X. Jia, C.Y. Oliver Chen,, J. B. Blumberg, Y. Song, W. Zhang, X. Zhang, G. Ma and J. Chen, 2007. Almond consumption reduces oxidative DNA damage and lipid peroxidation in Male smokers. J. Nutr., 137: 2717-2722.

Merkle, C.J., I.M. Moore, B.S. Penton, B.J. Torres, R.K. Cueny, R.C. Schaeffer and D.W. Montgomery, 2000. Methotrexate causes apoptosis in postmitotic endothelial cells. Biol. Res. Nurs., 2: 5-14.

Milosevic, J., S. Hoffarth, C. Huber and M. Schuler, 2003. The DNA damage-induced decrease of Bcl-2 is secondary to the activation of apoptotic effector caspases. Oncogene, 22: 6852-6856.

Miscia, S., A. Baldassarre and A. Cataldi, 1997. Shift of DNA polymerase a nuclear distribution and activity in apoptotic human leukaemia cells. Biochem. Biophys. Res. Commun., 234: 303-308.

Nassar, S., N. El-Ashmawy, S. El-Sawy, M. Attia and Y. Elhassaneen, 2003. Effect of aging and hormonal replacement therapy on rat testes: Links with apoptosis, mitochondrial function and oxidative stress. Egypt. J. Androl. Reprod., 17: 53-69.

Ohnishi, K., I, Ota, A. Takahashi and T. Ohnishi, 2000. Glecerol restores P53-dependent radiosensitivity of human head and neck cancer cells bearing mutant P53. Br. J., 83: 1735-1739.

Papaconstantinou, H.T., C. Xie, W. Zhang, N.H. Ansari, M.R. Hellmich, C.M. Townsend and T.C. Ko, 2001. The role of caspases in methotrexate-induced gastrointestinal toxicity. Surgery, 130: 859-865.

Raff, M.C., 1992. Social controls on cell survival and cell death. Nature, 356: 397-400.

Ricci, G., V. Pastukh, J. Leonard, J. Turrens, G. Wilson, D. Schaffer and S.W. Schaffer, 2007. Mitochondrial DNA damage triggers mitochondrial-superoxide generation and apoptosis. Am. J. Physiol. Cell. Physiol., 294: C413-C422.

Schwartz, D. and C. Cantor, 1984. Separation of yeast chromosomes by pulsed field gradient electrophoresis. Cell, 37: 67-75.

Williams, G.T. and C.A. Smith, 1993. Molecular regulation of apoptosis: Genetic controls on cell death. Cell, 74: 777-779.

Wlodek, D., J. Banath and P. Olive, 1991. Comparison between pulsed-field and constant-field gel electrophoresis for measurement of DNA double-strand breaks in irradiated Chinesehamster ovary cells. Int. J. Radiat. Biol., 60: 779-790.

Wyllie, A.H., 1995. The genetic regulation of apoptosis. Curr. Opin. Genet. Dev., 5: 97-104.

Xiao, G.F., Q. Lu and X.D. Yang, 2002. P73 gene expression in apoptotic process of acute myeloid leukemia cell line U937 induced by methotrexate. Zhongguo Shi Yan Xue Ye Xue Za Zhi, 10: 104-107.

Yoshida, H., Y.Y. Kong, R. Yoshida, A.J. Elia, A. Hakem, R. Hakem, J.M. Penninger and T.W. Mak, 1998. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell, 94: 739-750.