VIA MEDICA

ORIGINAL ARTICLE

Vol. 63, No. 4, pp. 407–412

ISSN 0015–5659
www.fm.viamedica.pl

Fast perinuclear clustering of mitochondria in oxidatively stressed human *choriocarcinoma* cells

Anna Hallmann[1], Ryszard Milczarek[1], Marcin Lipiński[2], Ewa Kossowska[2], Jan Henryk Spodnik[3], Michał Woźniak[4], Takashi Wakabayashi[5], Jerzy Klimek[1]

[1]*Department of Pharmaceutical Biochemistry, Medical University of Gdańsk, Poland*
[2]*Department of Biochemistry, Medical University of Gdańsk, Poland*
[3]*Department of Anatomy and Neurobiology, Medical University of Gdańsk, Poland*
[4]*Department of Medical Chemistry, Medical University of Gdańsk, Poland*
[5]*Department of Cell Biology and Molecular Pathology, Medical University of Gdańsk, Poland*

[Received 20 August 2004; Revised 21 September 2004; Accepted 21 September 2004]

Mitochondrial dysfunction plays a crucial role in cell types that exhibit necrosis-like death after activation of their death program. Tumour necrosis factor (TNF) induces abnormal, perinuclear clustering of mitochondria from an evenly spread distribution throughout the cytoplasm. The mitochondria withdraw from the cell periphery and aggregate in a unipolar perinuclear cluster. TNF-induced mitochondrial clustering is caused by impaired kinesin-mediated transportation of mitochondria. In this report, we describe a novel activity of menadione (MEN), namely the induction of an altered spatial distribution of mitochondria in the choriocarcinoma *JAR cells. Strikingly, 2 hours of cell exposition to menadione did not disrupt the integrity of the plasma membrane, while the intracellular ATP level significantly decreased. Control (untreated) cells displayed a typically scattered distribution of filamentary mitochondria inside the cell. After 2 hours of MEN treatment the spatial distribution of the mitochondria was markedly altered to an asymmetric perinuclear clustered distribution. Menadione-stressed cells displayed a highly asymmetrical perinuclear clustered distribution of the mitochondria. The exposure of cells to MEN also results in a change in shape of the mitochondria into a population of enlarged granular structures. The results of our study demonstrate that in JAR cells menadione causes mitochondria to translocate from the cell periphery into the perinuclear region several hours before disruption of cell membrane integrity and cell death.*

Key words: JAR cells, menadione, TNF, oxidative stress, mitochondrial translocation

INTRODUCTION

Recently accumulated evidence has shown that mitochondria display dual fundamental, albeit quite opposite, functions for eukaryotic cells. They are energy conserving organelles indispensable for their surveillance but can also be active players in programmed cell death [12]. Various pro-apoptotic stimuli are able to interfere with mitochondrial transmembrane potential, initiating irresistible commitment to cell death and preceding the late downstream events in apoptosis such as DNA fragmentation [17]. Mitochondria are now recognised as remarkably dynamic organelles. Time lapse microscopy of living cells provides evidence that mitochon-

Address for correspondence: Prof. Jerzy Klimek PhD, Department of Pharmaceutical Biochemistry, Medical University of Gdańsk, ul. Dębinki 1, 80–211 Gdańsk, Poland, tel/fax: +48 58 349 14 60, e-mail: klimekj@amg.gda.pl

dria undergo constant migration and profound morphological changes depending on frequent and continual cycles of mitochondrial fusion and fission [1, 19, 22, 23]. More importantly, mitochondrial mobility dynamics has been implicated in the regulation of apoptotic and necrotic phenomena initiated by membrane receptors for the tumour necrosis factor family, namely TNF and TRAIL. The mechanism of induction of apoptosis by TNF family members is initiated by a ligand binding to its target cell via high affinity plasma membrane surface receptors. Both TNF and TRAIL have been found to induce translocation of mitochondria from an evenly dispersed distribution to a clear cluster around the nucleus [5]. Attempts to identify precisely the downstream mechanism of ligand receptor interaction have suggested multiple intra-cellular pathways and the reported effects would appear to include an intact microtubule network [5] as well as the generation of reactive oxygen species [26]. The cytotoxic action of TNF does not depend on any great enhancement of RNA or protein synthesis by the inhibition of gene expression [24]. Analysis of the kinetics of the TNF-induced mitochondrial dynamic has shown perinuclear clustering preceding mitochondrial ROS production and subsequent cell death by several hours [24]. On the other hand, evidence has been provided that some cells may counteract cytotoxic TNF activity by the synthesis of rescue factors. Overexpression of mitochondrial MnSOD confers increased resistance to the cytotoxic effect of TNF [32]. These findings strongly imply that superoxide radicals might possibly participate in TNF-initiated mitochondrial translocation and cytotoxic signalling. We have previously shown that menadione (MEN) induces apoptosis and necrosis in human *choriocarcinoma* cells JAR (ATCC HTB-144) line [8]. It has also been reported recently that apoptosis induced by low doses of MEN is dependent on at least on one member of the TNF family of ligands, namely FAS-L [18, 25], involving activation of the NFκB transcription factor [3, 21], which is common for them, as well as Jun Kinase (JNK) and p38 MAPK pathways [11, 31, 33]. It has also been proposed by Laux et al. [13] that, while JNK activation and Fas-L expression play a role in cellular apoptosis at lower menadione concentrations, this effect yields to a predominant mitochondrial death mechanism at a higher drug concentration where the principal effect of MEN appears to be a failure of ATP synthesis, with little evidence for the involvement of caspases. Menadione metabolism is known to involve redox cycling of the parent quinone molecule and is 3-glutathionyl conjugate, resulting in the liberation of superoxide radicals [7]. In this report, we describe a novel activity of MEN, namely the induction of altered spatial distribution of mitochondria in *choriocarcionoma* cells. The effects of MEN on ATP level and the integrity of the plasma cell membrane were also compared.

MATERIAL AND METHODS

Cell line

Human *choriocarcinoma* cells JAR (ATCC HTB-144) were routinely cultured in 75-cm^2 plastic flasks (Sarsted) in RMPI 1640 containing 1 mM sodium pyruvate supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 100 IU/ml penicillin and 100 μg /ml streptomycin. The cells were grown in a humidified atmosphere with 5% CO_2 at 37°C. The cells were passaged weekly by trypsin 0.25% and EDTA 0.03%.

Reagents

Menadione sodium bisulfite (MEN) was a product of Sigma (USA). RMPI 1640 was also purchased from Sigma-Aldrich Chemicals (USA) and foetal bovine serum from Gibco BRL (Grand Island, NY, USA). Trypsin with EDTA and Phospate Buffer Saline (PBS) pH 7.4 were products of Sigma Chemicals (USA) and Mito Tracker Red CMXRos was produced by Molecular Probes, Inc. FITC — Phalloidin was a product of Sigma P5282 (USA). Formaldehyde and glutardehyde were purchased from Merck (Germany), Perma Fluor mounting solution from Immunon (Pittsburgh, PA, USA) and Triton X-100 from Sigma-Aldrich Chemicals (USA).

Treatment of cells with menadione (MEN)

Cells were plated in 6-well plates at a concentration of $5 \times 10^5 - 1 \times 10^6$ cells/well. The cells were treated with 50 μM menadione at specific times (0 h, 1 h, 2 h, 4 h, 24 h).

Viability assay

Cell viability was evaluated through lactate dehydrogenase (LDH) release. After substracting the relevant volume of medium (200 μl) from above the cells (to be used as reference in the following steps), the cells were lysed by the addition of Triton X-100 (final concentration in culture dish 1%). The aliquots (2 ml) of the lysed cells as well as the reference culture medium were centrifuged at 1500 $\times$ g for 3 min and supernatants were used for the assay.

10 μl of the assay solution was added to a cuvette containing 0.05 M Tris-HCl buffer, pH 7.4, 1 mM sodium pyruvate and 0.15 mM NADH, and spectrophotometrical analysis at 340 nm was performed. LDH release was expressed as a percentage of total release i.e. the release of LDH after cell lysis by 10% Triton X-100 [6].

Measurement of intracellular ATP level

Sample preparation: cells (~2 × 10^6 cells/ml) were collected by trypsinisation, centrifuged together with those floating in medium and washed with PBS. The cells were then suspended in 0.3 ml of cold 1.4 M $HClO_4$ and incubated for 15 min on ice to extract cellular nucleotides. Acid extracts (0.3 ml each) were centrifuged (14,000 RCF — 5 min), transferred to new tubes, and neutralised to pH ~6.5 by the addition of 0.1 ml of cold 3 M K_3PO_4 prior to centrifugation (14,000 RCF — 5 min). The supernatants were subjected to further analysis by HPLC. Protein concentration was evaluated using the Bradford method [2] after dissolving the perchloric acid precipitates with 0.5 M NaOH. The methods applied for HPLC determination of intracellular ATP level was based on that described by Smoleński et al. [29]

Staining and Visualisation of Mitochondria by Confocal Microscopy

Cells growing on 24 × 24 mm glass coverslips under various experimental conditions were incubated with 100 nM Mito Tracker Red CMXRos (CMXRos; Molecular Probes, Inc.) for 30 minutes at 37°C in an atmosphere of 5% CO_2. Cells were fixed with a fixative containing 2% glutaraldehyde and 2% formaldehyde in PBS for 30 minutes at room temperature and then washed in PBS and permeabilised with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. This was followed by 4 washes in PBS. The coverslips with cells were placed on glass slides in a Perma Fluor mounting solution (Immunon, Pittsburg, Pennsylvania) and analysed by the confocal system Radiance 2100 (Bio-Rad, UK), equipped with Krypton/Argon lasers and mounted on an Eclipse 600 (Nikon, Japan) microscope, using the software LaserSharp 2000 version 4.0 (Bio-Rad, UK) as previously described [10].

Results and Discussion

The cell viability assay revealed the cytotoxic activity of MEN after 24 hours of treatment. Nearly 50% of total LDH activity was detected in the extracellular medium. The intracellular ATP level as an index of the energy conserving process (pathway) fell dramatically to about 5% of the control value (control ~12 nmol/mg protein) (Fig. 1). Strikingly, 2 hours of cell exposure to menadione did not disrupt the integrity of the plasma membrane, while the intracellular ATP level significantly decreased (Fig. 1). Control (untreated) cells display a typically scattered distribution of filamentary mitochondria inside the cell (Fig. 2A). After 2 hours of MEN treatment the spatial distribution of the mitochondria was markedly altered to an asymmetric perinuclear clustered distribution (Fig. 2B). Menadione stressed cells displayed a highly asymmetrical perinuclear clustered distribution of mitochondria. The exposure of cells to MEN also results in a change in shape of the mitochondria into a population of enlarged granular structures.

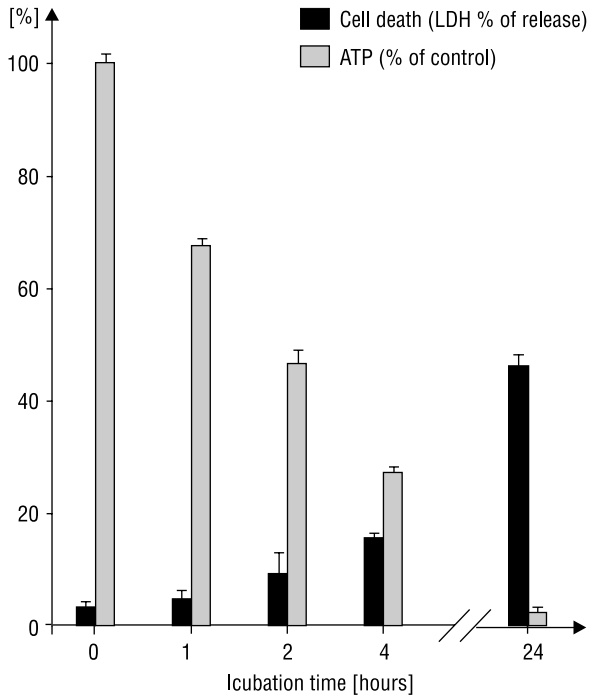

Figure 1. LDH release and the concentration of ATP in JAR cells after treatment with 50 μM menadione. The values represent means ± SEM from 3 independent experiments.

24 hours of treatment of *choriocarcinoma* cells with 50 μM menadione results in a non-apoptotic necrosis-like process characterised by disruption of the plasma membrane integrity and collapse of the energy conserving systems. Mitochondrial dysfunction plays a crucial role in cells types that display a necrosis-like death program. It has previously been demonstrated that disruption of the plasma membrane integrity by inflammatory cytokine TNF induces cell line L929 on the murine *fibrosarcoma*

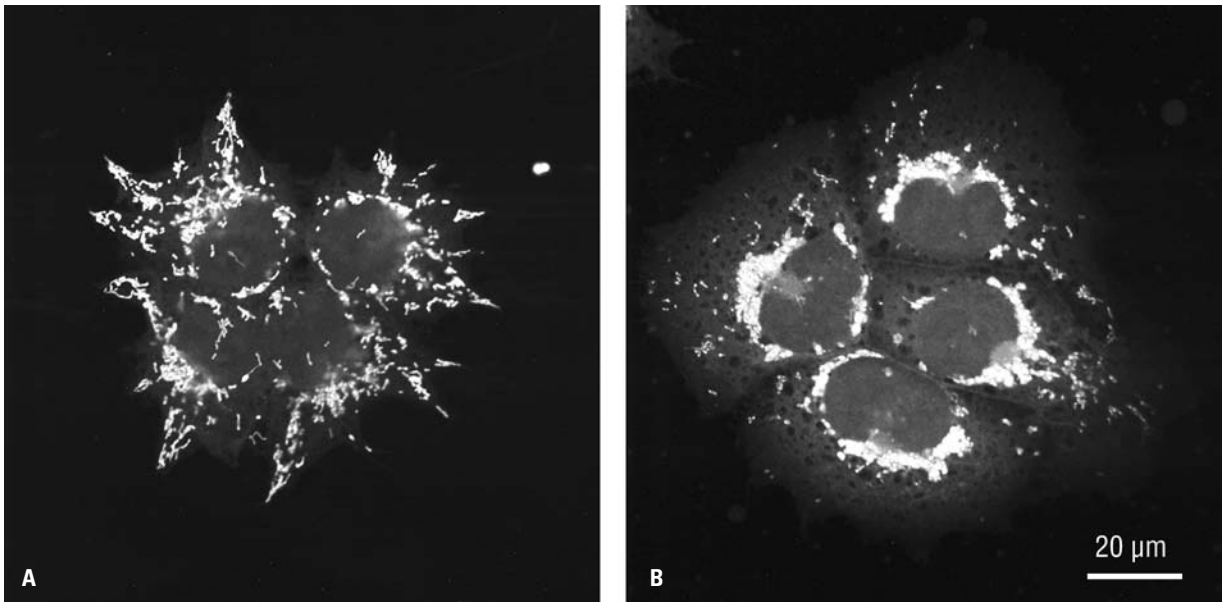

Figure 2. Distribution of mitochondria in JAR cells. **A.** Control cells; **B.** Treated with 50 μM menadione for 2 h.

[30]. In L929 cells, as well as in the human ovarian carcinoma cell line, there is evidence for an involvement of mitochondrial ROS in TNF-initiated cell death [28]. The most striking result of our study is its demonstration that MEN causes mitochondria to translocate from cells peripheral to the perinuclear region several hours before disruption of cell membrane integrity and cell death. As MEN is a well-known chemical source of superoxide anion generation inside the cell, the result demonstrates its ability to induce mitochondrial translocation points on an intrinsic feature of ROS so as to mediate a specific signal transduction pathway such as that displayed by TNF or TRAIL in other cell systems [14]. The spatial distribution of mitochondria is largely directed by two kinds of molecular motor, kinesin [27] and dynein [15]. Kinesin motor proteins are known to be plus-end directed, so kinesin-mediated organelle transport is predominantly directed to the cell periphery. Accordingly, loss of kinesin-mediated transport by the inhibitory effect of MEN will result in a perinuclear localization of the mitochondria as a result of the minus-end directed motor activity of dynein. Inactivation of kinesin by microinjection of an anti-kinesin antibody results in a clustering of mitochondria close to the nuclear region [16]. Since MEN induces a similar translocation of the mitochondria, we can speculate that the inactivation of kinesin in cells is a response to MEN. A viability assay revealed that clustering of the mitochondria is not a harmful phenomenon for the cell. We also observed distinct changes in the shape of the clustered mitochondria from the original elongated filamentous into distinctly enlarged granular ones. It is likely that these progressive morphological alterations in mitochondrial shape occur through mitochondrial fusion. The mitofusins Mfr1 and Mfr2 have been shown to promote mitochondrial fusion [4]. Our previous study revealed the important role of ROS in mediating changes in the shape of the mitochondria and their conversion to distinctly enlarged granular megamitochondria [9]. Such behaviour can be of great importance as a protective effect on the mitochondrial population in oxidatively stressed cells. The work of Ono et al. [20] has demonstrated that cells are protected from mitochondrial dysfunction by the complementation of DNA products in fused mitochondria. If such a mechanism is operative in *choriocarcinoma* cells oxidatively challenged with MEN, one may speculate that ROS-mediated mitochondria translocation and fusion is a part of a protective mechanism being converted to facilitate elimination of the cell if repair systems are ineffective. The perinuclear localization of the mitochondria facilitates the provision of ATP for repairing damaged nuclear components as well as intra-mitochondrially sequestered cell-death-inducing compounds.

REFERENCES

1. Bereiter-Hahn J, Voth M (1994) Dynamics of mitochondria in living cells: shape changes dislocation, fusion, and fission of mitochondria. Microsc Res Tech, 27: 198–219.
2. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72: 248–254.
3. Caricchio R, Kovalenko D, Kaufmann WK, Cohen PL (1999) Apoptosis provoked by the oxidative stress inducer menadione (Vitamin K3) is mediated by the Fas/ /Fas ligand system. Clin Immunol, 93: 65–74.
4. Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC (2003) Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. J Cell Biol, 160: 189–200.
5. De Vos K, Goossens V, Boone E, Vercammen D, Vancompernolle K, Vandenabeele P, Haegeman G, Fiers W, Grooten J (1998) The 55-kDa tumor necrosis factor receptor induces clustering of mitochondria through its membrane-proximal region. J Biol Chem, 273: 9673–9680.
6. Gallo M, Aragno M, Gatto V, Tamagno E, Brignardello E, Manti R, Danni O, Boccuzzi G (1999) Protective effect of dehydroepiandrosterone against lipid peroxidation in a human liver cell line. Eur J Endocrinol, 141: 35–39.
7. Gant TW, Rao DN, Mason RP, Cohen GM (1988) Redox cycling and sulphydryl arylation; their relative importance in the mechanism of quinone cytotoxicity to isolated hepatocytes. Chem Biol Interact, 65: 157–173.
8. Hallmann A, Masaoka M, Kamiński M, Klimek J, Woźniak M, Wakabayashi T (2003) Some characterization of human chorioncarcinoma cell line JAR cells against oxidative stress. 11th International Students' Scientific Conference, Gdańsk, 7.
9. Karbowski M, Kurono C, Wozniak M, Ostrowski M, Teranishi M, Nishizawa Y, Usukura J, Soji T, Wakabayashi T (1999) Free radical-induced megamitochondria formation and apoptosis. Free Radical Biology & Medicine, 26 (3–4): 396–409.
10. Karbowski M, Spodnik JH, Teranishi M, Wozniak M, Nishizawa Y, Usukura J, Wakabayashi T (2001) Opposite effects of microtubule-stabilizing and microtubule-destabilizing drugs on biogenesis of mitochondria in mammalian cells. J Cell Sci, 114: 281–291.
11. Kim BY, Han MJ, Chung AS (2001) Effects of reactive oxygen species on proliferation of Chinese hamster lung fibroblast (V79) cells. Free Radic Biol Med, 30: 686–698.
12. Kroemer G, Reed JC (2000) Mitochondrial control of cell death. Nature Med, 6: 513–519.
13. Laux I, Nel A (2001) Evidence that oxidative stress-induced apoptosis by menadione involves Fas-dependent and Fas- -independent pathways. Clin Immunol, 101: 335–344.
14. Lee YJ, Lee KH, Kim HR, Jessup JM, Sole DW, Kim TH, Billiar TR, Song YK (2001) Sodium nitroprusside enhances TRAIL-induced apoptosis via a mitochondria-dependent pathway in human colorectal carcinoma CX-1 cells. Oncogene, 20: 1476–1485.
15. Ligon LA, Tokito M, Finklestein JM, Grossman FE, Holzbaur EL (2004) A direct interaction between cytoplasmic dynein and kinesin I may coordinate motor activity. J. Biol Chem, 279: 19201–19208.
16. Lippincott-Schwartz J, Cole NB, Marotta A, Konrad PA, Bloom GS (1995) Kinesin is the motor for microtubule-mediated Golgi-to-ER membrane traffic. J Cell Biol, 129: 893 (Erratum in: J Cell Biol, 1995 Mar; 128 (5): following 988. J Cell Biol, 1995 May; 1995 Feb; 128: 293– –306).
17. Marchetti P, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A, Haeffner A, Hirsch F, Geuskens M, Kroemer G (1996) Mitochondrial permeability transition is a central coordinating event of apoptosis. J Exp Med, 184: 1155–1160.
18. Nishikawa Y, Wang Z, Kerns J, Wilcox CS, Carr BI (1999) Inhibition of hepatoma cell growth in vitro by arylating and non-arylating K vitamin analogs. Significance of protein tyrosine phosphatase inhibition. J Biol Chem, 274: 34803–34810.
19. Nunnari J, Marshall WF, Straight A, Murray A, Sedat JW, Walter P (1997) Mitochondrial transmission during mating in *Saccharomyces cerevisiae* is determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA. Mol Biol Cell, 8: 1233–1242.
20. Ono T, Isobe K, Nakada K, Hayashi JI (2001) Human cells are protected from mitochondrial dysfunction by complementation of DNA products in fused mitochondria. Nat Genet, 28: 272–275.
21. Osada S, Saji S, Osada K (2000) Critical role of extracellular signal-regulated kinase phosphorylation on menadione (vitamin K3) induced growth inhibition. Cancer, 91: 1156–1165.
22. Praefcke GJK, McMahon HT (2004) The dynamin superfamily: universal membrane tubulation and fission molecules? Mol Cell Biol, 5: 133–147.
23. Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, Tuft RA, Pozzan T (1998) Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. Science, 280: 1763– –1766.
24. Sanches-Alcazar JA, Schneider E, Hernandez-Munoz I, Ruiz-Cabello J, Siles-Rivas E, de la Torre P, Bornstein B, Brea G, Arenas J, Garesse R, Solis-Herruzo JA, Knox AJ, Navas P (2003) Reactive oxygen species mediate the down-regulation of mitochondrial transcripts and proteins by tumour necrosis factor-alpha in L929 cells. Biochem J, 370: 609–619.
25. Sata N, Klonowski-Stumpe H, Han B, Haussinger D, Niederau C (1997) Menadione induces both necrosis and apoptosis in rat pancreatic acinar AR4-2J cells. Free Radic Biol Med, 23: 844–850.
26. Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob W (1992) Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. J Biol Chem, 267: 5317–5323.
27. Seog DH, Lee DH, Lee SK (2004) Molecular motor proteins of the kinesin superfamily proteins (KIFs): structure, cargo and disease. J Korean Med Sci, 19: 1–7.
28. Siervo-Sassi RR, Marangoni AM, Feng X, Naoumova N, Winans M, Edwards RP, Lokshin A (2003) Physiological and molecular effects of Apo2L/TRAIL and cisplatin in ovarian carcinoma cell lines. Cancer Lett, 190: 61–72.

29. Smoleński RT, Lachno DR, Ledingham SJM, Yacoub MH (1990) Determination of sixteen nucleotides, nucleosides and bases using high-performance liquid chromatography and its application to the study of purine metabolism in hearts for transplantation. J Chromatogr, 527: 414–420.
30. Takahashi GW, Montgomery RB, Stahl WL, Crittenden CA, Valentine MA, Thorning DR, Andrews DF, Lilly MB (1994) Pentoxifylline inhibits tumor necrosis factor-alpha-mediated cytotoxicity and cytostasis in L929 murine fibrosarcoma cells. Int J Immunopharmacol, 16: 723–736.
31. Tuner NA, Xia F, Azhar G, Zhang X, Liu L, Wei JY (1998) Oxidative stress induces DNA fragmentation and caspase activation via the c-Jun NH2-terminal kinase pathway in H9c2 cardiac muscle cells. J Mol Cell Cardiol, 30: 1789–1801.
32. Wong GHW, Elwell JH, Oberley LW, Goeddel DV (1989) Overexpression of mitochondrial MnSOD confers increased resistance against cytotoxic effect of TNF. Cell, 58: 923–931.
33. Zhang X, Azhar G, Nagano K, Wei JY (2001) Differential vulnerability to oxidative stress in rat cardiac myocytes versus fibroblasts. J Am Coll Cardiol, 38: 2055–2062.