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The Reduced Coenzyme Nicotinamide Adenine Dinucleotide (NADH) repairs DNA damage of PC12 Cells induced by doxorubicin

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The effect of NADH on DNA repair was investigated on PC12 cells, damaged by doxorubicin. PC12 cells were incubated in medium without and with NADH before and after exposure to the DNA damaging agent doxorubicin. The changes of the cell proliferation genes (c-myc, c-erbB-2), the apoptosis inhibition gene bcl-2 and p53 (tumor suppressor gene), cell apoptosis gene (c-fos) and the proliferating cell nuclear antigen (PCNA) were investigated using a cytotoxicity assay and immunofluorescence flow cytometric analysis.

Doxorubicin induced DNA damage in PC 12 cells by inhibiting the expression of the cell proliferation genes and by triggering apoptotic processes in the cells. This was shown by down regulating the expression of c-erb-2, c-myc, bcl-2 and upregulating the expression of PCNA and c-fos of the PC 12 cells.

NADH did not only increase the resistance of PC 12 cells to the doxorubicin induced DNA damage but also repaired the damage partially. NADH promoted survival and differentiation by regulating the c-myc oncogene protein. Furthermore it supported the process of DNA repair by regulating the expression of p53 bcl-2 on the PC12 cells damaged by doxorubicin. NADH also down regulated expression of the cell apoptosis gene c-fos on the PC12 cells.

The expression of c-erbB-2 oncogene protein and PCNA on the PC12 cells did not show a significant change in the group of cells incubated with NADH in comparison to the group incubated with medium alone. In addition, an abnormal proliferation effect of NADH on PC12 cells has not been observed in these experiments.

For these results we conclude that NADH may be considered as a therapeutic adjunct for cancer patients to protect normal cells from the toxic effect of chemotherapeutic agents such as doxorubicin or cisplatin by stimulating the DNA repair system and by promoting normal cellular biosynthetic responses after chemotherapy.

The exposure of cells to DNA-damaging reagents can trigger a wide range of cellular responses involved in the regulation of gene expression and cell-cycle progression, stimulation of DNA repair and programmed cell death[1-2]. These

processes are important for maintaining normal growth, anti-mutation, damage repair and functional activity of cells. However, due to the unspecificity of chemotherapeutic drugs for the target cells, many normal cells in addition to the cancerous ones get damaged causing severe, sometimes fatal adverse reactions. The question is how can normal cells be protected from the cytotoxic effects of chemotherapeutic agents? How can we stimulate the repair system and promote normal cellular responses after chemotherapy? The mechanism involved in repairing DNA-damaged cells exposed to cytostatics has been investigated in many clinical studies.[1-5] However, whether the reduced form of the coenzyme nicotinamide adenine dinucleotide (NADH) can be used to protect cells from DNA-damage has never been considered. Previous studies in our laboratory have found that NADH can stimulate biosynthesis of endogenous cell factors, and can rescue cells from apoptotic damage by triggering production of the bcl-2 oncogene proteins[6]. In a different set of studies, NADH has been shown to improve symptoms of Parkinson's and Alzheimer's disease as well as of depression[7-11]. Even in a few cancer patients, NADH led to stabilization or improvement of disease[12]. The aim of this study was to elucidate the biological function and possible mechanism of NADH in repairing DNA-damage of PC12 cells induced by doxorubicin, a DNA-damaging reagent.[1,13,14] Using a cytotoxicity assay, altering immunocytofluorescence and flow cytometric analysis, the changes of the cell proliferation genes (c-myc, c-erbB-2), the apoptosis inhibition genes bcl-2, p53 tumor suppressor protein, the cell apoptosis gene (c-fos) and the proliferating cell nuclear antigen (PCNA) were investigated on PC12 cells before and after exposure to doxorubicin. Furthermore, the regulating activity of NADH on oncogen proteins on PC12 cells damaged by doxorubicin was studied. The potential mechanism by which NADH can repair DNA-damage is outlined.

Materials and methods

1. The cytotoxicity of doxorubicin for PC12 cells

48 hours before starting cytotoxicity test, rat pheochromocytoma cells (PC-12) were incubated as monolayer cells in 96 well tissue culture plate with RPMI-1640 medium (RPMI 1640 from Boehringer, Mannheim, Germany), 10% inactivated horse serum, 5% inactivated fetal calf serum, 1.2% glutamine (200mM), 1% penicillin and streptomycin (1 µg/ml) at 37° C, in 5% CO_2, 95% air and 95% humidity. Then cells were washed with RPMI-1640 medium and incubated for 4 and 10 hours in medium containing 0.0, 2.5, 5.0, 10.0, and 20.0 µg/ml of doxorubicin (Sigma D1515). The cytotoxicity of doxorubicin for PC12 cells was analyzed by colorimetric assay method [cell proliferation kit I, (MTT) Boehringer] according to routine procedure.[2,16]

2. Effect of NADH on the sensitivity of PC12 cells for cytotoxicity of doxorubicin

In order to investigate the action of NADH on regulating cells resistant to chemotherapy reagents, PC12 cells were firstly incubated for 48 hours in RPMI-1640 medium without and with 400 µg/ml NADH in 75 cm^3 tissue culture flasks (Szabo, Vienna, Austria). Then the cells were washed with RPMI-1640 medium, and then incubated in medium containing 10 µg/ml of doxorubicin for 4, 6 and 10 hours in 96 well tissue culture plates respectively. The cell proliferation activity of PC12 cells was analyzed with MTT method as above.[2,16]

3. Measurement of the repair activity of NADH on repairing PC12 cells with a DNA-damage induced by doxorubicin

PC12 cells were grown as monolayer, incubated in RPMI-1640 medium mixed with 20 μg/ml of doxorubicin for 4 hours in 96 well tissue culture plates, were then washed with medium. Then they were incubated in medium alone or medium containing different concentration of NADH (50-600 μg/ml) for 48 hours. The cell proliferation activity was measured by MTT method for evaluating the effect of NADH on repairing DNA damage of PC12 cells. In another series of tests, PC12 cells were incubated first in medium containing doxorubicin (0.05 mM/ml and 0.15 mM/ml) for 4 hours, and then incubated in medium with 400 μg/ml of NADH for 48 hours. The cell proliferation activity was analyzed as above.[2,16]

4. Flow cytometric evaluation of the effect of NADH on regulating cellular response to doxorubicin damage

The PC12 cells were incubated in RPMI-1640 medium with 10% heat inactivated horse serum, 5% inactivated fetal calf serum and 1.2% 200mM glutamine in 162 cm^2 tissue culture flasks. The culture medium was changed every three days until the cells grew as monolayers[2,16]. The cells were then washed with medium and incubated in medium without (normal group) and with 10 μg/ml doxorubicin for 10 hours. The 5×10^6 PC12 cells damaged by doxorubicin were harvested, washed with PBS twice and stored as actual damage group. Subsequently, these were washed and further incubated 48 hours in the medium without and with 400 μg/ml of NADH for 48 hours in new 75-cm^2 tissue culture flasks. The cells were trypsinized, washed with PBS twice, followed by fixation of the cell pellets in 0.5% paraformaldehyde at 4° C for 10 minutes. Then the fixed cells were treated with 0.1% Triton X-100 for making the cellular and nuclear membranes permeable according to the routine procedure. For analysis of the antigen and genetic markers, the cells were added into each test tube to 300 μl of the cell suspension (up to 2.5×10^5 cells in each tube). The cells were washed twice with 4 ml of PBS, and centrifuged at 300xg for 5 minutes. The supernatant was aspired. The antibodies against P53 tumor suppressor protein (clone DO-7), c-myc oncoprotein (clone C-33), bcl-2 oncoprotein (clone 124), c-erbB-2 oncogene protein (clone NCL-CB11), c-fos (UB-06341) and proliferating cell nuclear antigen (PCNA, clone PC10) were added to each tube respectively, mixed, and incubated with PC12 cells for 1 hour at 37° C. The cells were washed twice with 4 ml of PBS, and centrifuged at 300 xg for 5 minutes at room temperature. The supernatant was aspired. Added in 50 μl of monoclonal antibody against mouse (Sigma F-8771, 1:200) or polyclonal antibody against rabbit conjugated with FITC (Neomaker Co 1:500), mixed and incubated in the dark for 30 minutes at 37° C. Mouse monoclonal antibodies were replaced by normal serum as the negative control. After washing twice with 4 ml of PBS, supernatants were discarded by aspiration and cells were detached from the pellet by mild vortexing. The cells were resuspended in 0.3 ml PBS and 10000 events were analyzed on a flow cytometer. The immunofluoresence amount and the positive ratio of antibodies recognized on the vital and apoptotic PC12 cells were measured by computerized flow cytometry.[17,18,19]

Results

1. NADH increase cellular resistance of PC12 cells to DNA-damage

After PC12 cells have been incubated in medium containing doxorubicin (2.5, 5.0, 10.0, 20.0 μg/ml) for 4 and 10 hours, a significant inhibition of the proliferation acti-

vity of PC12 cells was observed. The degree damage of PC12 cells was set in correlation to exposure time and concentration of doxorubicin incubated with cells (10 hours, IC_{50} concentration was 10ug/ml) is given in (Fig 1). However, when PC12 cells were preincubated with NADH two days prior to doxorubicin exposure, the sensitivity of PC12 cells for the cytoxocity of doxorubicin was found to be down-regulated to 10,3 ± 3.1 % (Fig 2). This observation indicates that NADH can increase the resistance of PC12 cells to DNA-damage induced by doxorubicin.

2. NADH can partially repair DNA-damage of PC12 cell induced by doxorubicin

The results of the cell proliferation activity assay show that the inhibition rate of PC12 cells was 23.3%, after exposure to doxorubicin at concentration of 20 μg/ml for 4 hours. The continuously damaging effect of doxorubicin on PC12 cells can be repaired by incubation with NADH. The repair activity of NADH on DNA-damage of PC12 cells depends on the concentration of NADH in medium (Fig 3). As can be seen of (Fig 4), the degree of damage of PC12 cells influences the effect of NADH on the repairing of the DNA damage.

The cytometric analysis revealed for the exposure of PC12 cells to doxorubicin (10 μg/ml) for 10 hours, revealed a ratio of apoptotic to normal PC12 cells 51.2 ± 7.5 %. After incubation for 48 hours, the ratio of vital cells was 17.7 ± 5.6% in the group incubated with medium alone and 20.8 ± 5.8% in the group with NADH. The ratios of apoptotic PC12 cells in lag phase were found to be 82.2% and 79.2% respectively. The repair ratio of NADH on PC12 cell with DNA-damage was 3.1% in comparison to the group incubated with medium alone. This observation also indicates that the ability of NADH to rescue PC12 cells from apoptosis appears to be dependent on the degree of cell damage of these cells.

3. Doxorubicin induces cell apoptosis by down-regulation of the expression of proliferation oncogene on PC12 cells

Immunofluoresence flow cytometric analysis found the amount of c-erbB-2, c-myc, P53, Bcl-2 expressed on the PC12 cells damaged by doxorubicin in acute phase 53.3%, 97.8%, 91.9%, and 98.8% respectively in comparison to normal cells. However, the amount of c-fos expressed on the PC12 cells was upregulated to 94.1% after incubation with doxorubicin (Fig 5). These findings suggest that doxorubicin induces DNA-damage of cells by inhibiting expression of cell proliferation genes and by triggering apoptotic processes in PC12 cells.

4. NADH down-regulates expression of c-fos on PC12 cells damaged by doxorubicin

After recovery culture for 48 hours, amount of c-fos oncogene proteins expressed on vital PC12 cells were found to be down-regulated 65.0% in the presence of NADH and 47.7% in medium alone, in comparison with acutal stage of damage. The positive ratio of c-fos expressed on PC12 cells was 16.9% and 25.3% in NADH and medium alone groups (Fig 6-7). Indicating that NADH may inhibit apoptotic processes associated with c-fos oncogen expression.

5. Effect of NADH on regulating c-myc and c-erbB-2 expressed on PC12 cells damaged by doxorubicin

After exposure to doxorubicin, PC12 cells were incubated in medium without and with NADH for 48 hours. The results of immunofluorescence flow cytometric analysis showed that the ratio of the vital to apoptotic PC12 cells was 6.78 ± 0.7% in medium alone group and 8.41 ± 2.8% in NADH group. A lag

phase of apoptotic damage by doxorubicin on the PC12 cells was observed after incubation with medium only and NADH respectively. The repair ability of NADH on DNA-damage seem to be dependent on the degree of damaged PC12 cells in acute phase. Cytometric analysis indicates that NADH can repair early damage and block lag damage of c-myc gene on PC12 cells. The amount of c-myc oncogene protein expressed on PC12 cell increases to 52.9% and 47.4% under the influence of NADH in comparison to medium group and acute phase of damage. The amount of c-erbB-2 expressed on PC12 cells was found not to be significantly different between NADH group and medium group ($P > 0.05$) (Fig 8-9). However, the positive rates of c-erbB-2 and c-myc expressed on PC12 cells were significantly lower in NADH group (15.4% and 12.6%) than in the control group (26.01% and 23.4%).

6. Effect of NADH on the expression of Proliferation Cell Nuclear Antigen (PCNA) in DNA repair

The results of immunofluorescence and flow cytometry assay indicate the positive ratio of PCNA expressed on the vital PC12 cells to be 90.8% in the acute damage phase of doxorubicin and 69.6% in normal cells. However, the exposure of PC12 cells to doxorubicin resulted in a significant increase in the immunofluorescence associated with nuclear-bound PCNA, in comparison to normal cells (Fig 5). After incubation with medium and NADH for 48 hours, the amount of PCNA expressed on the vital PC12 cells still showed a higher level (Fig 9). The positive ratio of PCNA was found to be decreased by incubating PC12 cells for 48 hours. But there was no significant difference between the group of cells incubated with medium only and medium containing NADH (Fig 8).

7. Effect of NADH on bcl-2 and p53 expressed on PC 12 cells damaged by doxorubicin

The PC12 cells damaged by doxorubicin (IC: 82.8%) were incubated in medium with NADH for 48 hours. The amount of the tumor suppressor protein p53 on the total PC12 cells increased by 40.2% and 55.7% in comparison to the group containing only medium and acute damaged phase (Fig 10). Further analysis indicated that the increased production of p53 was mainly found to be in the apoptotic cells. It increased by 52.4% on the apoptotic cells in the presence of NADH in comparison to the cells incubated with medium alone. However, the amount of p53 expressed on the vital PC12 was decreased 36.7% in the NADH group (Fig 11).

These findings suggest that NADH can block apoptotic damage induced by doxorubicin in lag phase. NADH may be an essential co-factor in the repair process of DNA-damage by down-regulating the tumor suppressor protein expression. The production of bcl-2, the apoptosis inhibiting protein, on the PC12 cells was upregulated to 96.7% after incubation with medium and equally high after incubation with NADH. The total amount of bcl-2 expressed on the cells did not differ between the medium and NADH group ($P>0.05$), but it was found to be increased by 52.4% on the apoptotic cells in comparison with only medium group (Fig 10). However, the amount of bcl-2 expressed on the vital cells in NADH group was found to be increased 12.7% and 22.9% in comparison to medium alone group in the acute damage phase (Fig 11).

Discussion

1. Doxorubicin induces the apoptosis of PC12 cell by blocking oncogene expression and activation of c-fos

Drug-induced apoptosis is dependent on the balance between cell cycle checkpoints and DNA repairing mechanisms. Doxorubicin

is a DNA-damaging cytotoxicic drug, which is found to accumulate in the nuclei of damaged cells. Increased accumulation of cellular doxorubicin is accompanied by apoptosis[1,2,13]. Our experiments indicate that the inhibition rate of PC12 cells correlates with the concentration of doxorubicin in medium and with time of exposure of the cells to the toxic environment (Fig 1-2).

The cytotoxicity of doxorubicin for PC12 cells occurs not only in the phase of acute exposure but also in the lag phase.

Doxorubicin induces apoptosis in PC12 cells, which is accompanied by the down-regulation of the expression of the oncogene proteins c-erb-2 and c-myc, the anti-apoptotic gene proteins (bcl-2), p53 tumor suppressor protein and upregulation of the expression of PCNA [3] and c-fos (Fig 5).

These genetic changes occur not only in the early phase of the apoptosis induced by doxorubicin, but can also happen in the lag phase, when the damaged PC12 cells are incubated with new medium after removing the old medium containing doxorubicin (Fig 7-8). DNA damage and activation of c-fos oncogen seem to be the major pathways of inducing apoptotic damage of PC12 cells.

2. NADH can partial rescue cell activity of PC12 cell from DNA damage induced by doxorubicin

Cell damage repair is a complex biological process in which a number of reactions are involved. It has been widely investigated in various systems such as DNA damage reversal, base excision repair, nucleotide excision repair and mismatch repair.[1-5]

NADH is an essential component of enzymes necessary for many metabolic reactions in the cell including energy production. It plays a crucial role in triggering biological antioxidation and in regulating the expression of membrane glycoprotein receptors. [20,21] Our previous studies have shown that NADH can rescue cells from apoptosis caused by inhibition of the mitochondrial respiratory chain induced by chemotherapic agents, and simultanously can increase the production of endogenous biological factors necessary for proper functions. In addition, cell cycle progression of PC12 cells is observed[2,6].

The question is can NADH repair DNA damage? If the answer is yes, we want to know how it regulates gene expression involved in DNA repair. The results of our cytotoxicity test and flow cytometric assays indicate that the repair ability of NADH on damaged cell depends on the degree of DNA-damage of PC12 cells (Fig 4).

When the apoptotic ratio of PC12 cell was 82.2%, the rate of cells repaired by NADH was only 3.1%. After recovery incubation for 48 hours, the expression of c-erbB-2 oncogene proteins and PCNA on the PC12 cells did not show a significant increase in the group treated with NADH in comparison with medium alone group.

The significantly abnormal proliferation effect of NADH on PC12 cells was not shown in this experiment. The change of cerbB-2 oncogen happening in the acute damage phase of the PC12 cells is difficult to be rescued by incubation with NADH or medium. However, the upregulation of c-fos oncogene protein in the acute damage phase can be significantly down-regulated by incubation with NADH for 48 hours (Fig 7). This suggests that NADH rescues PC12 from doxorubicin induced damage not only by repairing the DNA but also by increasing energy production in these cells. Our observations provide substantial evidence that treating patients with NADH will not induce abnormal proliferation of cells.[6,8,9]

3. NADH can regulate the expression of c-myc oncogen protein on PC12 cells with DNA-damage

Programmed cell death is an energy dependent biochemical regulated process

that is the result of the expression of a number of genes. The roles of several gene and gene families such as Bcl-2/bax, P53, c-myc, c-jun, c-fos, considered to be critical for apoptosis have recently been described in different cell lines.[23,24] Many reports suggest that a rather complex genetic and molecular mechanism is involved in the process of apoptosis. It could also be triggered either by increased or by reduced gene expression as well as by biochemical reactions not necessarily connected to altered gene expression.[1, 22,23,24,25]

Further investigations are necessary to understand the molecular mechanism by which NADH may repair the DNA-damage in cells. Further insight into this process may lead to the discovery of new therapeutic strategies targeted to counteract degeneration and apoptosis of cells.

Our observations provide evidence that complex molecular events are involved in the apoptotic process of PC-12 cells induced by doxorubicin. After recovery incubation of PC-12 cells with NADH for 48 hours, the positive ratio and amount of c-erbB-2 expressed on PC12 cells did not show an increase in comparison to the control with medium alone group. The positive ratio of c-myc was not altered, but the amount of c-myc expressed on the vital PC12 cells was significantly upregulated 47.4% and 52.9% in comparison to the acute damage phase and the group with medium alone (Fig 9). This suggests that regulating the expression of c-myc on PC12 cells may be involved in the DNA repair of PC12 cells damaged by doxorubicin. Although the exact function of c-myc remains largely unknown, its activation has been implicated in the induction of cell proliferation, and differentiation. Some reports have also indicated that the c-myc oncogene protein acts as sequence-specific transcription factor that serve to regulate gene expression in normal cellular growth and differentiation and as a common intracellular transducer which promote G_0 to G_1 transition. They may also be involved in the regulation of programmed cell death[27,28,29,30]. Our results indicate that c-myc is involved in the action of NADH on the repairing of doxorubicin-induced DNA-damage by promoting normal growth and differentiation, at least in the PC12 cells regarded as model for neuron cells.

4. NADH repairs DNA-damage of PC12 cells by regulation of p53 and bcl-2 gene protein expression

In the processes of cell DNA damage and repair, bcl-2 and p53 are the two of the most important proteins encoded by the bcl-2 gene and p53 tumor suppressor gene. Wild-type p53 can suppress cell proliferation and slow DNA synthesis and block transition from G_1 to S phase of the cell cycle.[30,31,32] Bcl-2 is a proto-oncogene and the most important inhibitor of apoptosis. Expression of bcl-2 may interfere with the apoptotic process mediated by the APO-1/Fas antigen and TNF receptor. Probably the ratio of bcl-2 and p53 determines how the cell responds to DNA-damaging agents. Current research indicates that expression of bcl-2 in pheochromocytoma cells is associated with that of the c-myc oncogen protein.[34,35,36] Over-expression of the proto-oncogen bcl-2 might block p53-induced apoptosis and inhibit p53 functional activity.[37] In our experiment, in which we investigated the effect of NADH on the recovery of PC12 cell from DNA-damage, the ratio of expression of p53 and bcl-2 on PC12 cells was down-regulated by 91.9% and 98.8% by exposure of the cells to doxorubicin in medium. After recovery incubation in medium with NADH for 48 hours, the ratio of vital PC12 cells was upregulated by 3.1% and p53 tumor suppressor protein expressed on the vital cells down-regulated 36.7%. However, the amount of bcl-2 expressed on the vital PC12 cells was found to be upregulated by 12.7% in comparison to the control group (medium alone). These findings suggest that NADH

can not only promote survival and differentiation by regulating the c-myc oncogene protein, but also support the process of DNA repair by regulating the expression of p53 tumor suppressor protein and proto-oncogen protein bcl-2 on the PC12 cells damaged by doxorubicin.

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References

1. Wang JY. Cellular responses to DNA damage. Curr Opin Cell Biol 1998; 10(2): 240-247.
2. Stecca C, Gerber GB. Adaptive response to NDA-damaging agents: a review of potential mechanisms. Biochem Pharmacol 1998; 55(7): 941-951.
3. Savio M, Stivala LA, Bianchi L, Vannini V, Prosperi E. Involvement of the proliferation cell niclear antigen (PCNA) in DNA repair induced by alkylating agents and oxidative damage in human fibroblasts Carcinogenesis 1998; 19(4): 591-596.
4. Anderson CW. Protein kinases and the response to DNA damage. Semin Cell Biol 1994; 5(6): 427-436.
5. Fink D, Aebi S, Howell SB. The role of DNA mismatch repair in drug resistance. Clin Cancer Res 1998 4(1): 1-6.
6. Zhang JR, Vrecko K, Nadlinger K, Storga D, Birkmayer GD, Reibnegger G. The Reduced Coenzyme Nicotinamide Adenine Dinucleotide (NADH) rescues PC12 cells from apoptosis. J Tumor Marker Oncology 1998; 13(3): 11-24.
7. Birkmayer GJD, Birkmayer W. Stimulation of endogenous L-dopa biosythesis-a new principle for the therapy of Parkinson's disease: the clinical effect of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotidephosphate (NADPH). Acta Neurol Scan. 1989; 126: 183-187.
8. Birkmayer JGD, Vrecko C, Volc D, Birkmayer W. Nicotinamide adenine dinucleotide (NADH) - a new therapeutic approach to Parkinson's disease, comparison of oral and parenteral application. Acta Neurol. Scand. 1993; 87 (Suppl 146): 32-35.
9. Birkmayer GD, Birkmayer W. The coenzyme nicotinamide adenine dinucleotide (NADH) as biological antidepressive agent experimence with 205 patients. New Trends in Clinical Neuropharmacology 1991; 5: 75-86.
10. Birkmayer JGD. The new therapeutic approach for improving dementia of the Alzheimer type. Ann Clin Lab Sci 1996; 26: 1-9.
11. Storga D, Vrecko K, Birkmayer JGD, Reibnegger G. Monoaminergic neurotransmitters, their precursors and metabolites in brains of Alzheimer patients. Neuroscience Letters 1996; 203:29-32.
12. Birkmayer JGD. Nicotinamide Adenine Dinucleotide (NADH) - a new therapeutic approach: Preliminary results with cancer patients and patients with dementia of the Alzheimer type. Journal of Tumor Marker Oncology 10(1), 1995:71-78.
13. Hideki K, Yoshihiro M, Michihiko K, Ikuo M, Masaji H, Hiroyuki S, Shichisaburo A. Direct measurement of doxorubicin concentration in the intact, living single cancer cell during hyperthermia. Cancer 1997; 79: 214-219.
14. Ramachandran C, You W, Krishan A. Bcl-2 and mdr-1 gene expression during

doxorubicin-induced apoptosis in murine leukenic p388 and p388/R84 cells. Anticancer Research 1997; 17(5A): 3369-3376.
15. Doxorubicin-induced apoptosis in human T-cell leukemia is mediated by caspase-3 activation in a Fas-independent way FEBS LETTERS 1997; 417(3): 360-364.
16. Mosmann T. Rapid clorimetric assay for cellular growth and survuval: Aplication to proliferation and cytotoxicity assays. J. Immunol Methods 1983; 65(1-2): 55-63.
17. Boersma AW, Nooter K, Burger H, Kortland CJ, Stoter G. Bax upregulation is an early event in cisplatin-induced apoptosis in human testicular germ cell tumor cell line NT2, as quantitated by flow cytometry. Cytometry 1997; 27(3): 275-282.
18. Gong J, Traganos F, Darzykiewicz Z. Discrimination of G2 and mitotic cells by flow cytometry based on different expression of cyclin A and B1. Exp Cell Res 1995; 220(1): 226-231.
19. Stuter L, Bobadilla M, Koch E, Bechter R. Flow cytometric evaluation of the effects of doxorubicin on rate sperma-togenesis. Reprod Toxicol 1997; 11(4): 521-531.
20. Alberts B, Bray D, Lewis J, Raff H, Roberts K, Watson JD. Energy conversion: mitochondria and chloroplasts. Molecular Biology of the Cell, 3rd edtion. Garland Publishing Inc. 1994; 653-720.
21. .Marques F, Bicho MP. Activation of a NADH dehydrogenase in the human erythrocyte by beta-adrenergic agonists: possible involvement of a G protein in enzyme actvation. Biol Signals 1997; 6(2): 52-61.
22. Macaya A. Apoptosis in the nervous system. Rev Neurol 1996; 24 (135): 1356-1360
23. Cherbonnel-Lasserre C, Dosanjh MK. Suppression of apoptosis by over-expression of Bcl-2 or Bcl-xL promotes survival and mutagenesis after oxidative damage. Biochimie 1997; 79(9-10): 613-617.
24. Huang DC, O'Reilly LA, Strasser A, Cory S. The anti-apoptosis function of Bcl-2 can be genetically separated from its inhibitory effect on cell cycle entry. EMBO J 1997 16(15): 4628-4638.
25. Lovschall H, Kassem M, Mosekilde L. Apoptosis: Molecular aspects. Nord Med 1997; 112(8): 271-275.
26. Hopewell R, Li L, MacGregor D, Nerlov C Ziff EB. Regulation ofcell proliferation and differentiation by Myc. J Cell Sci Suppl 1995;19: 85-89.
27. Depinho RA, Schreicer AN, Alt FW. Myc family oncogenes in the development of normal and neoplastic cells. Adv Cancer Res 1991; 57: 1-45.
28. Luscher B and Eisennman RN. New light on myc and myb. Part 1, myc. Genes and Development. 1990; 4: 2025-2035.
29. Kangas A, Nicholson DW, Hittla E. Involvement of CPP32/Caspase-3 in c-myc-induced apoptosis. Oncogene 1998; 16(3): 387-398.
30. Hughes PE, Alexi T, Schreiber SS. A role for tumour suppressor gene p53 in regulating neuronal apoptosis . Neuroreport 1997; 8(15): 5-12.
31. Blagosklonny MV, Ei-Deiry WS. Acute overexpression of wt p53 facilitates anticancer drug-induced death of cancer and normal cell. Int J Cancer 1998; 75(6): 933-940.
32. Findley HW, Gu L, Yeager AM, Zhou M. Expresson and regulation of bcl-2, bcl-xl, and bax correlate with p53 status and sensitivity to apoptosis in childhood acute lymphoblastic leukemia. Blood 1997; 89(8): 2986-2993.
33. Da-Gong W, Colin FJ, John JM, Kerry VP, Brew A, Colin FJR, and Keith DB.

Expression of the apoptosis-suppressing gene bcl-2 in pheochromocytoma is associated with the expression of c-myc. J Clinical Endocrinology & Meta. 1997; 82 (6): 1949-1952.
34. Egle A, Villunger A, Marschitz I, Kos M, Hittmair A, Lukas P, Grunewald K, Greil R. Expression of Apo-1/fas CD95), Bcl-2, Bax and bcl-x in myeloma cell lines: relationship between responsiveness to anti-Fas mab and p53 functional status. Br J haematol 1997, 97(2): 418-428.
35. Katoh S, Mitsui Y, Kitani K, Suzuki T. The rescuing effect of nerve growth factor if the result of up-regulation of bcl-2 in hyperoxia-induced apoptosis of a subclone of pheochromocytoma cells PC12h. Neurosci Lett 1997; 232(2): 71-74.
36. Birchall MA, Schock E, Harmon BV, Gobe G. Apoptosis, mitosis, PCNA and bcl-2 in normal, leukoplakic and malignant epithelia of the human oral cavity: prospective, in vivo study. Oral Oncol 1997; 33(6): 419-425.
37. Heidenreich A, Schenkman NS, Sesterhenn IA, Mostofi KF, Moul JW, Srivastava S, Engelmann UH. Immunohistochemical and mutational analysis of the p53 tumour suppressor gene and the bcl-2 oncogene in primary testicular germ cell tumours. APMIS 1998; 106(1): 90-99.

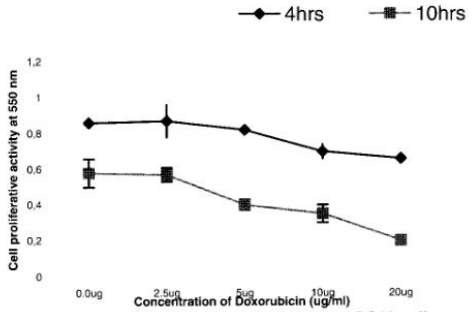

Fig 1. Cytotoxicity of doxorubicin on PC12 cells

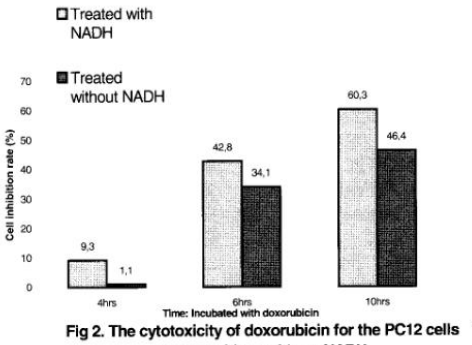

Fig 2. The cytotoxicity of doxorubicin for the PC12 cells treated with or without NADH

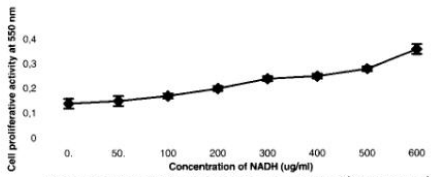

Fig3. Relationship between the recovery of damage of PC12 cells and concentration of NADH. (PC12 cells incubated with doxorubicin for 4 hours and then with NADH for 48 hours)

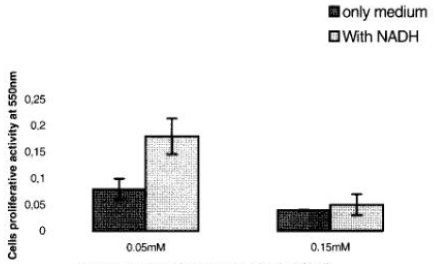

Fig 4. Relationship between the repair of NADH on damaged PC12 cells and the concentration of doxorubicin

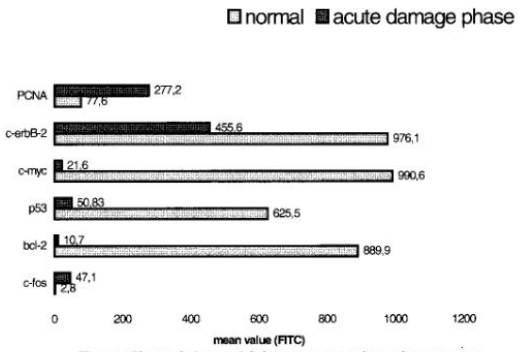

Fig. 5 effect of doxorubicin on expression of oncogene proteins on PC12 cells

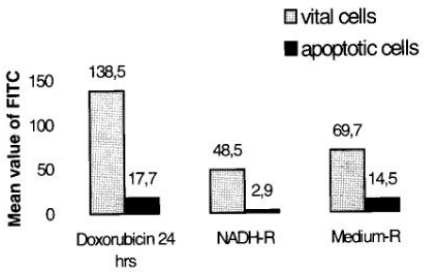

Fig 6. NADH regulates the expression of c-fos on PC12 cells damaged by doxorubicin

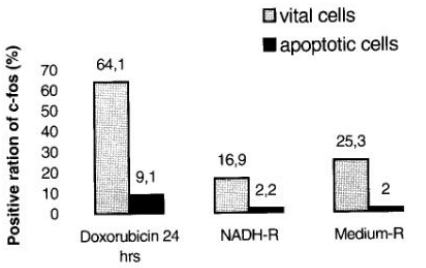

Fig7. NADH downregulates the expression of c-fos on PC12 cells damaged by doxorubicin

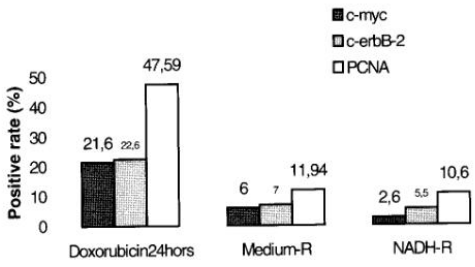

Fig 8. Effect of NADH on c-myc. c-erbB-2 and PCNA expressed on PC12 cells damaged by Doxorubicin

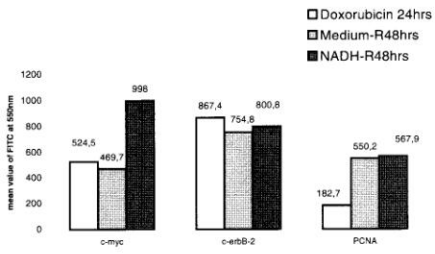

Fig 9. NADH regulates the expression of c-myc, c-erbB-2 and PCNA on the vital PC12 cells damaged by doxorubicin

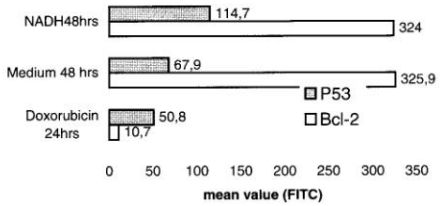

Fig 10. NADH regulates the expression of Bcl-2 and P53 on PC12 cells damaged by doxorubincin

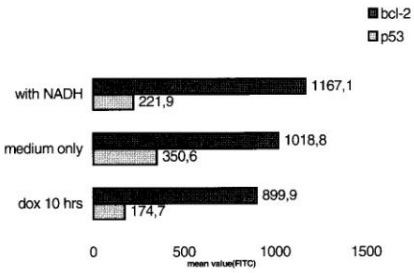

Fig 11. Effect of NADH on p53 and bcl-2 expressed on the vital PC12 cells damaged by doxorubicin