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Antioxidative and immunomodulatory role of melatonin, sodium selenite, *N*-acetyl-L-cysteine and quercetin on human umbilical blood

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We have previously reported on the DNA oxidative damage and oxygen stress in healthy term neonates. The aim of the present study was to investigate the antioxidative and immunomodulatory role of melatonin, sodium selenite, *N*-acetyl-L-cysteine and quercetin on umbilical blood. The single cell gel electrophoresis (comet) assay was used for DNA oxidative damage. The lymphocytes proliferation and natural killer (NK) activity in umbilical blood were assayed by ^{3}[H]-thymidine uptaken and MTT methods. Results using comet assay showed protection by melatonin, *N*-acetyl-L-cysteine (NAC) and quercetin, and a protective and damaging effect by selenite sodium. Melatonin, sodium selenite, NAC and quercetin greatly promoted the lymphocytes proliferation to IL-2. NK activity of the umbilical blood was significantly increased by melatonin and sodium selenite, but was not affected by NAC and quercetin. These data suggest that antioxidants are able to protect umbilical blood mononuclear cells against oxygen stress and affect oxygen stress mediated immune function inhibition of umbilical blood. These results will supply new experimental data for using umbilical blood to treat diseases.

1. Introduction

The process of childbirth is accompanied by an increase in oxidative aggression. Many studies have shown that at birth the neonate presents an increase in the rate of free radical generation and/or a decline in the competence of antioxidants. Therefore, tissues of neonates are relatively more vulnerable to free radical damage (Muller et al. 1987; Neefjes et al. 1999; Roblers et al. 2001). Our previously report have shown the DNA oxidative damage in umbilical blood mononuclear cells and oxygen stress in healthy term neonates (Zhao et al. 2004).

Many reports have shown that immune cells are very sensitive to oxygen stress and antioxidants have immunomodulating action improving altered immune function (Bendich 1992; Del Rio et al. 1998). Thus, antioxidants play a vital role maintaining immune cells in a reduced environment and protect them from oxidative stress (McArthur et al. 2000). Therefore, *in vitro*, the function of immune cells can probably be modulated by antioxidants. In addition, neonates have a weaker immune function, due to the relative immune immaturity of lymphocytes (Gaddy et al. 1995; Racadot et al. 1993). However, a correlation between the lower immune function of neonates and oxygen stress during birth has not been established.

Melatonin, *N*-acetyl-L-cysteine (NAC), quercetin and sodium selenite (Na_2SeO_3), have been shown to have antioxidative properties (Cadenas et al. 1999; Tan et al. 1994; Rotruck et al. 1973; Ganther et al. 1986; Aruoma et al. 1989; Beckman et al. 1996; Zhou et al. 1991; Manach et al. 1996; Chen et al. 1990). Quercetin has been shown, by our group, to have the antioxidative ability (Liu et al. 2002; Zhao et al. 2002). Moreover, we have observed that melatonin may facilitate the repair of the damaged DNA of tumor-associated lymphocytes in malignant pleural effusion (Liu et al. 2003). The immunomodulatory roles of these antioxidants also have been reported. In humans, an increase of lymphocyte proliferation was observed with melatonin (Garcia-Maurino et al. 1999). Selenium has been shown to increase NK cells and cytotoxic lymphocyte viability in mice (Spetrie et al. 1989) and rats (Koller et al. 1986). NAC supplementation increased NK activity of old mice (Ferrandez et al. 1999). However, little is known about the antioxidative and immunomodulatory effects of these antioxidants on human umbilical blood.

Thus, the aim of the present study was to investigate whether antioxidants have the ability to protect umbilical blood mononuclear cells against oxygen stress and affect oxygen stress mediated immune function inhibition of umbilical blood.

2. Investigations and results

2.1. Protection of antioxidants against DNA damage induced by H_2O_2

The comet assay was used to determine the protective effects of antioxidants on DNA damage. Melatonin produced a significant reduction in DNA damage at concentrations from 0.1–100 μM but reduced DNA damage more effectively at 1 μM than at other concentrations. Selenite sodium significantly reduced DNA damage at concentrations from 0.87–3.4 μM. Higher concentrations of 6.8 μM had no significant effect on DNA damage, but

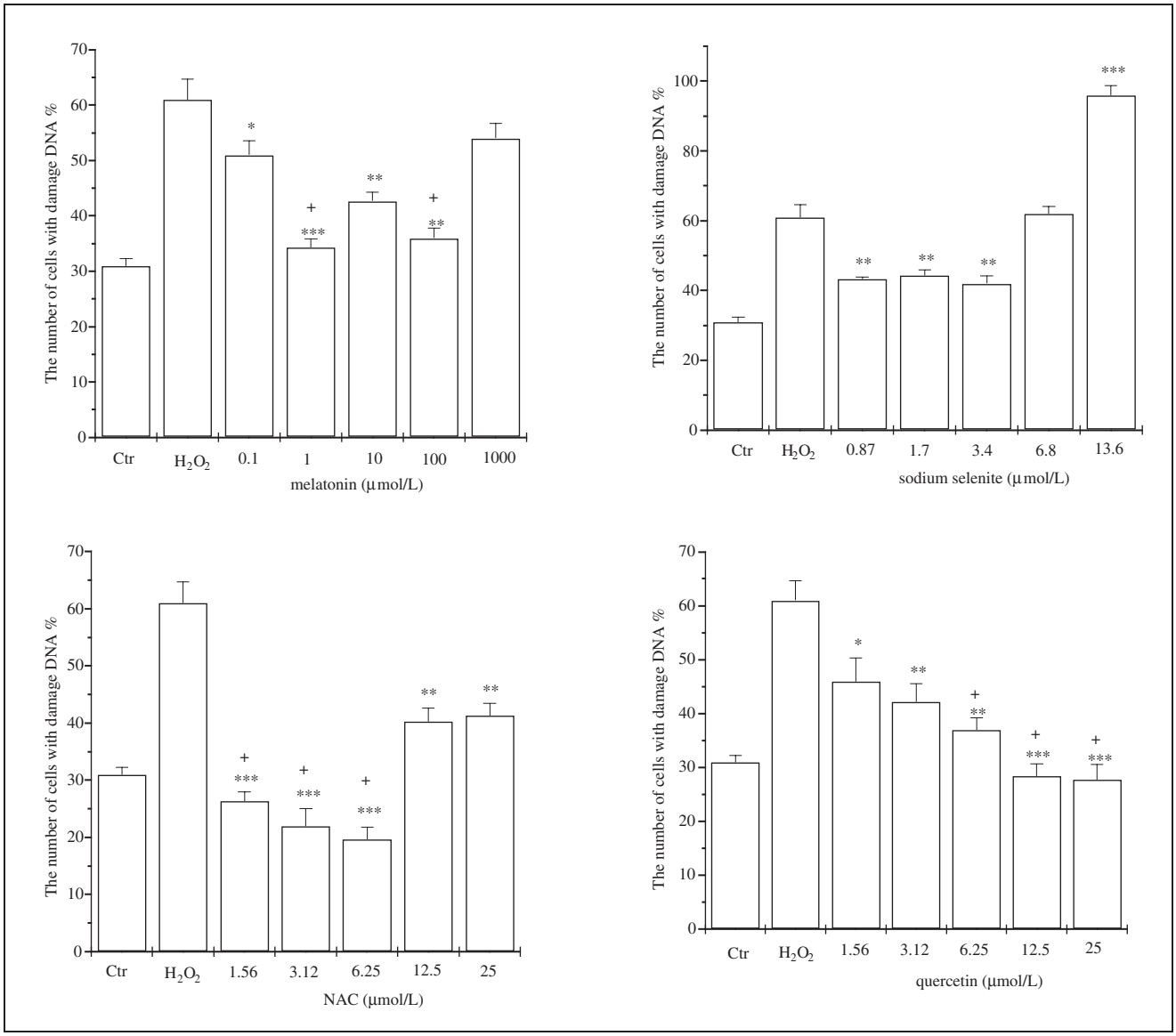

Fig. 1: The effect of supplementation *ex vivo* with different concentrations of antioxidants on H_2O_2-induced DNA damage of umbilical blood mononuclear cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with H_2O_2 alone treated. + $p > 0.05$ compared with control

13.6 μM actually increased the damage caused by incubation with H_2O_2. NAC and quercetin produced a significant reduction in DNA damage after the cells had been damaged with hydrogen peroxide at concentrations from 1.56 to 25 μM. For NAC, concentrations from 1.56 μM to 6.25 μM seemed to offer the best protection. The protection by quercetin at concentrations from 12.5 to 25 μM was more effective than at other concentrations (Fig. 1). These results show that antioxidants are effective to protect oxidative DNA damage from oxygen stress.

2.2. Antioxidants affected the lymphocytes proliferation and NK activity of umbilical blood

After 48 h and 72 h incubation with different concentrations of antioxidants, 2×10^5/well mononuclear cells were harvested and the lymphocytes proliferation were detected with ^{3}[H]-thymidine uptake methods as described in the experimental section. Fig. 2 shows the proliferation of umbilical blood mononuclear cells to IL-2 in the presence or absence of various antioxidants. At 48 h, lymphocytes proliferation of IL-2 was significantly stimulated by 1.74 μM sodium selenite. At 72 h, 10–100 μM melatonin, 0.87–3.48 μM sodium selenite, 1.25–5 μM NAC and 5–10 μM quercetin greatly promoted the lymphocytes proliferation to IL-2.

After incubation with K562 cells for 18 h, NK activity was assayed by MTT methods. NK activity was greatly increased by 1–100 μM melatonin and 0.87–3.48 μM sodium selenite. 10 μM melatonin and 1.74 μM sodium selenite were more effective in increasing NK activity than other concentrations. NK activity was not affected by NAC or quercetin (Fig. 3). These antioxidants had no effect on the growth of K562 cells themselves (data not shown).

These data suggest that antioxidants have the ability to improve altered immune function of the umbilical blood.

3. Discussion

Our data show a direct protective activity of melatonin against H_2O_2-induced DNA damage. Melatonin, an endogenously indolamine formed in adult humans, is a highly effective antioxidant and free radical scavenger. Previous studies have shown that melatonin protects against oxida-

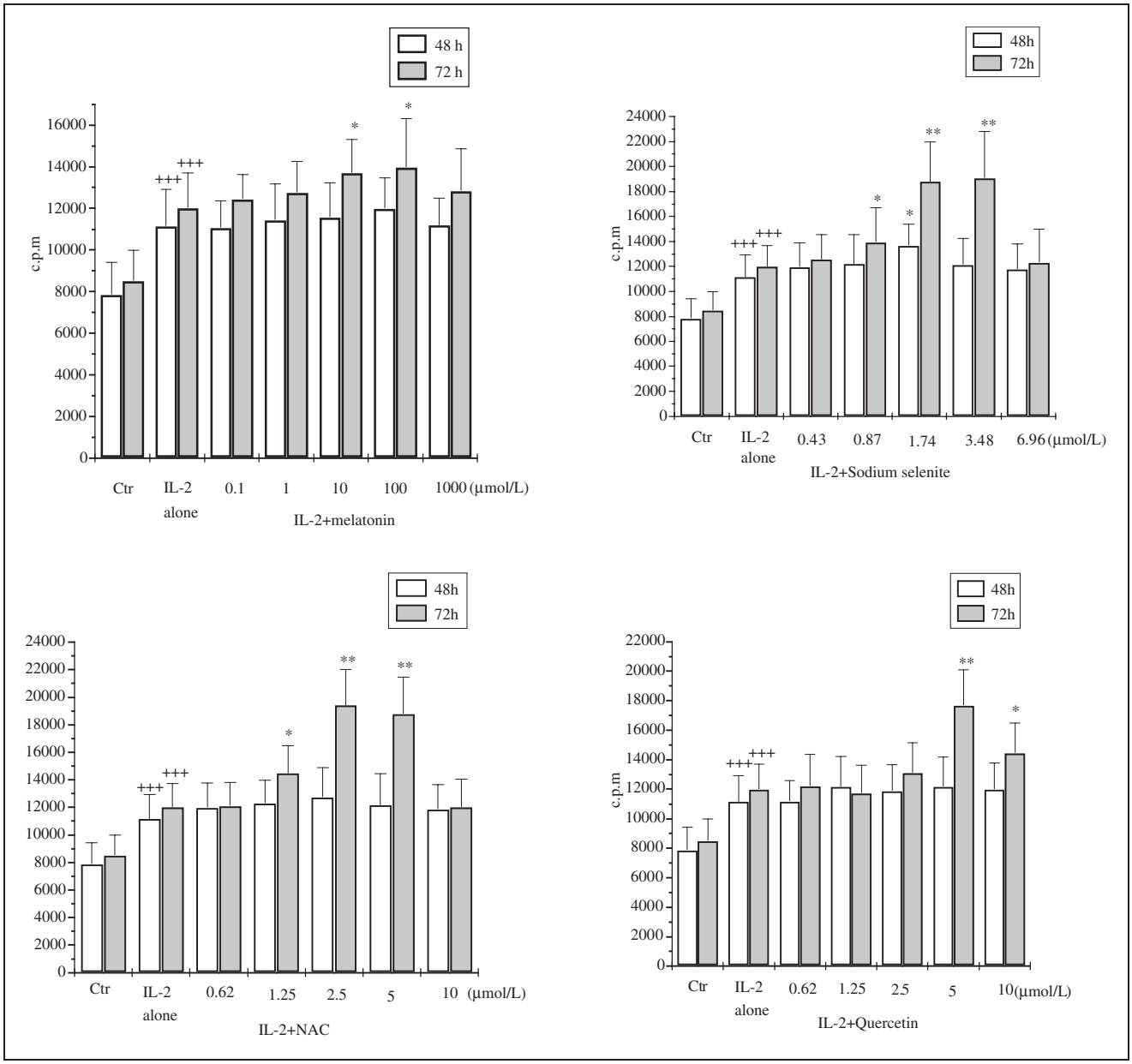

Fig. 2: Umbilical blood mononuclear cells proliferation in response to IL-2 only, and in combination with various antioxidants at different concentrations and times. (+) Significantly different to control values ($^{+++}$ $P < 0.001$). (*) Significantly different to values stimulated with only IL-2 (* $P < 0.05$, ** $P < 0.01$). n = 12

tive DNA damage in animals challenged with oxidative stress, e.g. melatonin can prevent the oxidative DNA damage induced in the kidney by the carcinogen $KBrO_3$ (Cadenas et al. 1999) and melatonin also prevented hepatic DNA from oxidative damage following the administration of the chemical carcinogen safrole (Tan et al. 1994). Selenium, as a component of the glutathione peroxidase, is an important antioxidant (Rotruck et al. 1973). Our results show selenite sodium to protect DNA from damage at lower concentrations, however, at higher concentrations, the damage caused by incubation with H_2O_2 increases. It was proposed that selenium toxicity is related to changes in intracellular concentration of reduced glutathione (GSH), and excess selenium has been shown to interact with cellular sulfhydryls. Selenium in the oxidant selenite undergoes reductive metabolism, utilizing GSH and NADPH as a source of reducing equivalents (Ganther et al. 1986). NAC also has antioxidant properties. As a sulfhydryl donor, it may contribute to the synthetic precursor of intracellular cysteine and GSH. NAC may have additional protective ability to reduce oxygen radical-related oxidant processes by directly interfering with the oxidants, up-regulating antioxidant systems such as superoxide dismutase (Aruoma et al. 1989; Beckman et al. 1996). Quercetin, one of the most prevalent and thoroughly studied dietary flavonoids, may interfere with oxidative processes both by chelating metal ions, scavenging of hydroxyl and peroxy radicals and inhibiting 15-lipoxygenase and LDL oxidation (Zhou et al. 1991; Manach et al. 1996; Chen et al. 1990). The present study show a strong protecting effect of NAC and quercetin against DNA damage. All these data demonstrated that antioxidants have the ability to protect umbilical blood mononuclear cells against oxygen stress.

The results from our research also suggest that antioxidants affect the immune function of umbilical blood. This

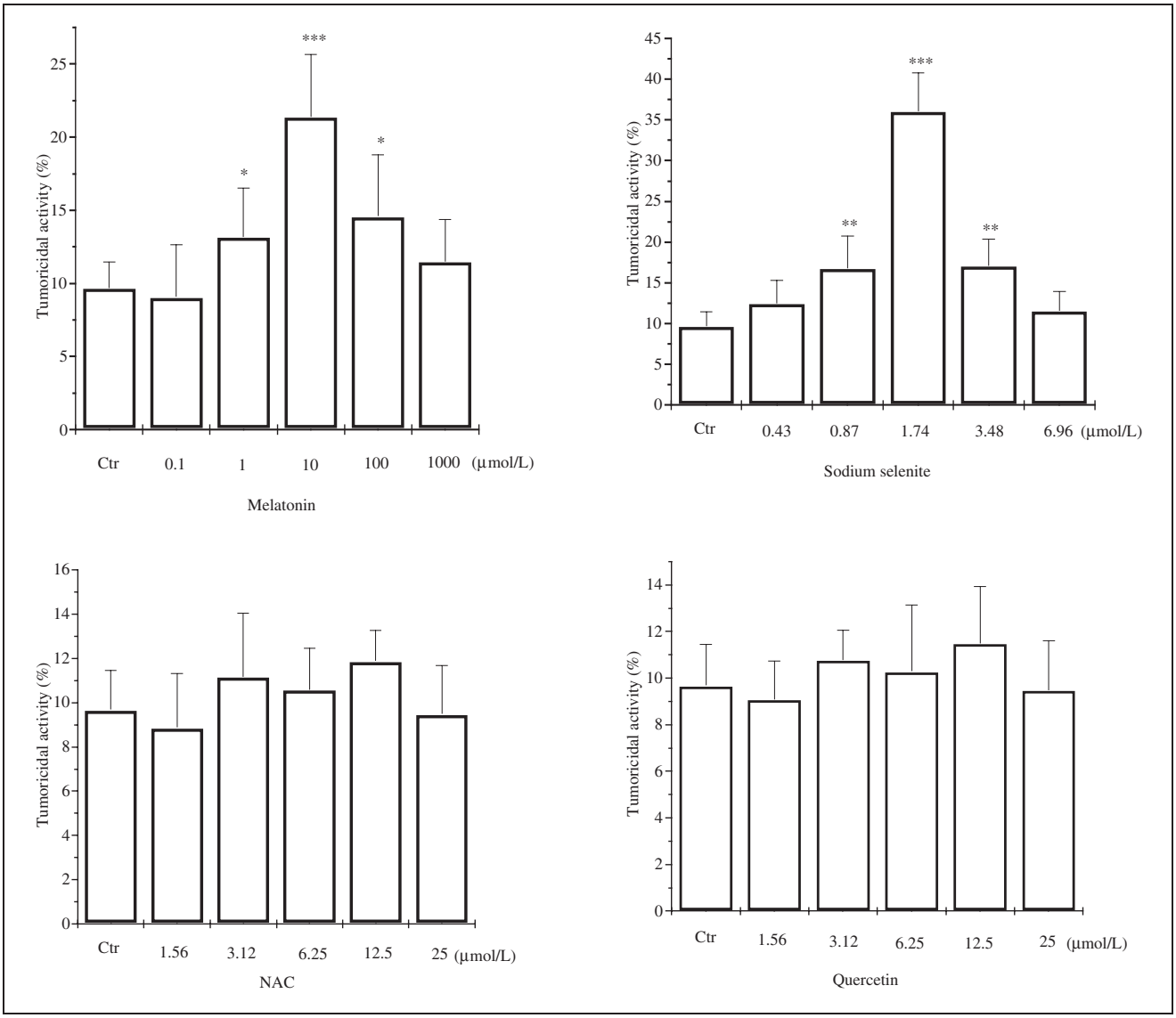

Fig. 3: Effect of various antioxidants at different concentrations on the killing activity of NK cells towards K562 cells. The results are expressed as the tumoricidal activity. (*) Significantly different to control values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). $n = 6$

finding agrees with the generally accepted idea that immune cells are very sensitive to oxygen stress and antioxidants have the ability to improve altered immune function. The present study showed *in vitro* the beneficial effects of melatonin on lymphocytes proliferative response to IL-2 and NK activity of human umbilical blood. *In vivo* supplementation with melatonin is reported to promote lymphocyte proliferation (Garcia-Maurino 1999). Some of the antitumor effects of melatonin could be related to increased NK cell cytotoxicity. Melatonin could increase NK cells cytotoxicity via an increased production both IL-2 and IL-12 (Garcia-Maurino 1999). Our findings that selenite sodium increased lymphocytes proliferative response and NK activity were consistent with others. Selenium deficiency significantly inhibited the ability of the lymphocytes to proliferate in response to mitogens, whereas selenium supplementation significantly enhanced this response. Supplementation with selenium resulted in a significant increase in NK cell number and NK activity of mice (Kiremidjian-Schumacher et al. 1996, 1998). The favourable effect of NAC on lymphocyte function have been already described by other authors and may be due to both it's antioxidative role and some specific metabolic actions as a thiolic compound. NAC modulates immune function through different pathways, such as increasing lymphocyte proliferation, IL-2 production and IL-2 receptor expression (Aruoma et al. 1989; Omara et al. 1997). These results are consistent with ours that NAC promoted lymphocytes proliferative response. However, in our study, quercetin increased lymphocyte proliferative response which is in contrast to other studies showing inhibition (Mookerjee et al. 1986). Probably in our study, quercetin increased the lymphocytes proliferative response by regulating the oxygen stress status of umbilical blood mononuclear cells caused by birth. Our results show that NAC and quercetin did not affect NK activity. This implies that another mechanism may be involved in this process. Above data indicate that the lower immune function of umbilical blood maybe related with oxygen stress.

In other words, antioxidants are effective in resistant oxygen stress and preserve adequately immune function of umbilical blood. These results will supply new experimental data for using umbilical blood therapeutically.

4. Experimental

4.1. Reagents

Melatonin, *N*-acetyl-L-cysteine (NAC), quercetin, MTT (3-[4,5-dimetylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), low and normal melting point agarose (LMA, NMA), Triton-X-100, ethidium bromide, were purchased from Sigma Chemical Company. Sodium lauroyl sarcosine was produced by Amresco. Culture medium (RPMI-1640) was obtained from Gibco Laboratories. Calf serum was purchased from Si-Ji-Qing Biotechnology Company (Hangzhou, China). Lymphocyte separation medium and selenite sodium were produced by Shanghai Second Reagent Company (Shanghai, China). All other reagents were of analytical purity.

4.2. Methods

4.2.1. Isolation of umbilical blood mononuclear cells

Human umbilical blood was obtained from the Obstetrics and Gynaecology Hospital of Lanzhou and ethical approval for the study was granted. Informed consent was obtained from all the mothers. In this study, blood samples were collected from healthy full term neonates. Mononuclear cells were separated using lymphocyte separation medium, washed in PBS and suspended in culture medium RPMI-1640 supplemented with 10% heat-inactive calf serum, benzylpenicillin 100 U/ml, and streptomycin 100 μg/ml in a 5% CO_2 atmosphere at a concentration of 2×10^6/ml cells. Cell viability was tested using trypan blue dyes exclusion. The cell viability was more than 90%.

4.2.2. Antioxidant pre-treatment and H_2O_2 challenge

All the antioxidants were diluted to the required concentrations using PBS. Cells were incubated with different concentrations of antioxidants for 20 min in a dark incubator together with untreated control samples. Then H_2O_2 (final concentration 25 μM) was added to the medium which incubated for 10 min at 4 °C. Control samples were treated with PBS alone without H_2O_2. Samples were then centrifuged at 1000 × g for 10 min and the cells washed with PBS.

4.2.3. Comet assay

Reactive oxygen species (ROS) can cause DNA damage. Comet assay is a rapid and sensitive technique for analysis of DNA breakage in single mammalian cells. The comet assay has been used in various studies to investigate the effect of ROS on DNA, and the protective effects of antioxidants (Collin et al. 1995; Duthie et al. 1997).

DNA damage, measured by the comet assay, was performed as described by Singh (1988) with minor modification. 25 μl mononuclear cell suspension were mixed with 50 μl 1% low melting point agarose at 37 °C, and then placed on a microscope slide which precoated with 0.5% normal melting point agarose and covered with a coverglass. The slides were kept at 4 °C for 5 min for solidification of the agarose. After removal of the coverglass, the slides were immersed in lysing solution (1% sodium lauroyl sarcosine, 2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, pH 10, and 1% Triton-X-100, 10% DMSO added freshly) for 70 min to lyse the cells and to permit DNA unfolding. The slides were then placed in an electrophoretic buffer (1 mM Na_2-EDTA and 300 mM NaOH) for 20 min for DNA unwinding followed by 20 min electrophoresis at 34 V and 310 mA. These electrophoresis conditions had been identified in prior experiments as a suitable range of DNA damage in adult peripheral blood mononuclear cells.

All of the steps described above were conducted in subdued light to prevent DNA damage from UV light. Afterwards, the slides were washed with cold neutralizing buffer (0.4 M Tris, pH 7.5) twice, dried gently and stained with 25 μl of 20 μg/ml ethidium bromide each slide, followed by covering the slide with a coverglass.

Cells were viewed at × 200 magnification with a fluorescent microscope (BH-2 OLYMPUS, excited by green light and a barrier filter of 590 μm) within 24 h. Photomicrographs were taken using a Lucky black and white film ASA 400 (Xiamen, China). For each sample three slides were scored, with at least 50 cells counted randomly on each slide. Cells without damaged DNA retain a circular appearance. DNA strand breaks in cells migrates towards the anode during electrophoresis, the cell appears as a comet. Cells with damaged DNA were graded by eye into two categories corresponding to the length of the comet tail. Comet was defined as having a tail longer than the diameter of comet head according to Everett (2000). So the percentage of comet (the comet %) reflected the level of DNA damage in cells.

4.2.4. Target cells

A human erythroleukemic cell line (K562 cells), was obtained from Shanghai Cell Biology Institute, Chinese Academy of Sciences. The cells were cultivated at 37 °C in an atmosphere containing 5% CO_2. The culture medium was RPMI-1640 supplemented with 10% heat-inactivated calf serum, benzylpenicillin 100 U/ml, and streptomycin 100 μg/ml.

4.2.5. Lymphocyte proliferation assay

Various antioxidants (melatonin, sodium selenite, NAC and quercetin), with or without 100 U/ml interleukin-2, were added and the cultures, with mononuclear cells (2×10^5 cells/well), were incubated in 96-well culture plates for 48 h and 72 h in a humidified incubator containing 5% CO_2 at 37 °C. Mononuclear cells were then pulsed with 2 μCi/well of ^{3}H-thymidine for 4 h before being harvested with a harvester. Mononuclear cell proliferation, as determined by ^{3}H-thymidine uptake, was assessed with a β-counter.

4.2.6. NK activity assay

To obtain a significant NK activity, the different effector/target rate were used. As a result, mononuclear cells and K562 cells ratios used-40/1-allowed us to observe significant results. Mononuclear cells (4×10^5/well), K562 cells (1×10^4 /well) or then mixtures were cultured for 18 h in the presence or absence of various antioxidants. Then the culture medium was removed and 100 μl per well of MTT (2 mg/ml) dye solution was added. Cell proliferation was assayed by reduction of MTT. The percentage of tumoricidal activity was calculated according to the formula previously described (Hruby and Beck 1997):

$$\text{Activity } (\%) = \left(1 - \frac{\text{OD (mononuclear cells + K562 cells)} - \text{OD (mononuclear cells alone)}}{\text{OD (K562 cells alone)}} \right) \times 100 \quad (1)$$

The effect of antioxidants on tumor cell growth was calculated as:

$$\text{Activity } (\%) = \frac{\text{OD (K562 cells + antioxidant)}}{\text{OD (K562 cells alone)}} \times 100 \quad (2)$$

4.2.7. Statistics

The data ($\overline{X} \pm SD$) were analyzed for statistically significance by two-tailed t test for paired data, using the SPSS/PC +4 statistical package (SPSS Inc, Chicago, IL). Difference was considered significant at $P < 0.05$.

References

Aruoma OI, Halliwell B, Hoey BM, Butler J (1989) The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. J Free Radic Biol Med 6: 593–597.

Beckman JS (1996) Oxidative damage and tyrosine nitration from peroxynitrite. Chem Res Toxicol 9: 836–844.

Bendich A (1992) Vitamins and immunity. J Nutr 122: 601–603.

Cadenas S, Barja G (1999) Resveratrol, melatonin, vitamin E, and PBN protect against renal oxidative DNA damage induced by the kidney carcinogen $KBrO_3$. Free Radic Biol Med 26: 1531–1537.

Chen YT, Zheng RL, Jia ZJ, Ju Y (1990) Flavonoids as superoxide scavengers and antioxidants. Free Radical Bio Med 9: 19–21.

Collins AR, Ma AG, Duthie SJ (1995) The kinetics of repair of oxidative DNA damage (strand breaks and oxidized pyrimidines) in human cells. Mutat Res 336: 69–77.

Del Rio M, Ruedas G, Medina S, Victor VM, De la Fuente M (1998) Improvement by several antioxidants of macrophage function *in vitro*. Life Sci 63: 871–881.

Duthie SJ, Collins AR, Duthie GG, Dobson VL (1997) Quercetin and myricetin protect against hydrogen peroxide-induced DNA damage {strand breaks and oxidized pyrimidines} in human lymphocytes. Mutat Res 393: 223–231.

Everett SM, White KLM, Schorah CJ, Calvert RT, Skinner C, Miller D, Axon ART (2000) *In vivo* DNA damage in gastric epithelial cells. Mutat Res 468: 73–85.

Ferrandez MD, Correa R, Del Rio M, De La Fuente M (1999) Effects *in vitro* of several antioxidants on the natural killer function of aging mice. Experim Gerontol 34: 675–685

Gaddy J, Risdon G, Broxmeyer HE (1995) Cord blood natural killer cells are functionally and phenotypically immature but readily respond to interleukin-2 and interleukin-12. J Interferon Cytokine Res 15: 527–536.

Ganther HE (1986) Pathways of selenium metabolism including respiratory excretory products. J Am Coll Toxicol 5: 1–5.

Garcia-Maurino S, Pozo D, Carrillo-Vico A, Calvo JR, Guerrero JM (1999) Melatonin activates Th1 lymphocytes by increasing Il-12 production. Life Sci 65: 2143–2150.

Hruby Z, Beck K-F (1997) Cytotoxic effect of autocrine and macrophage-derived nitric oxide on cultured rat mesangial cells. Clin Exp Immunol 107: 76–82.

Kiremidjian-Schumacher L (1998) Selenium and immune function. Z Ernahrungswiss 37: 50–56.

Kiremidjian-Schumacher L, Roy M, Wishe HI, Cohen MW, Stotzky G (1996) Supplementation with selenium augments the functions of natural

killer and lymphokine-activated killer cells. Biol Trace Elem Res 52: 227–239.
Koller L, Exon J, Talott P, Osborne C, Woi H (1986) Immune response in rats supplemented with selenium. Clin Exp Immunol 63: 570–576.
Liu GA, Jin FL, Zheng RL (2002) Protection against damaged DNA in single cell by polyphenols. Pharmazie 57: 852–854.
Liu XJ, Zhao J, Zheng RL (2003) DNA damage of tumor-associated lymphocytes and total antioxidant capacity in cancerous patients. Mutat Res 539: 1–8.
Manach C, Regerat F, Texier O, Agullo G, Demigne C, Remesy C (1996) Bioavailability, metabolism and physiological impact of 4-oxo-flavonoids. Nutr Res 16: 517–544.
McArthur WP (2000) Effect of aging on immunocompetent and inflammatory cells. Periodontol 16: 53–79.
Mookerjee BK, Lee TP, Lippes HA, Middleton E Jr (1986) Some effects of flavonoids on lymphocyte proliferative responses. J Immunopharmacol 8: 371–392.
Muller DPR (1987) Free radical problems of the newborn. Proc Nutr Soc 46: 69–75.
Neefjes VEM, Evelo CTA, Baars LGM, Blanco CE (1999) Erythrocyte glutathione S transferase as a marker of oxidative stress at birth. Arch Dis Child Fetal Neonatal Ed 81: F130–133.
Omara FO, Blakley BR, Bermier J, Fournier M (1997) Immunomodulatory and protective effects of *N*-acetylcysteine in mitogen-activated murine splenocytes *in vitro*. Toxicology 116: 219–226.
Racadot E, Schaal JP, Van Lemmens P, Billot M, Herve P (1993) Immunological characteristics of umbilical cord blood cells: phenotypic and functional analysis. J Hematother 2: 251–253.
Roblers R, Palomino N, Robles A (2001) Oxidative stress in neonate. Early Hum Dev 65: S75–81.
Rotruck JT, Pope AL, Ganther HE, Hafeman DG, Swanson AB, Hockstra WG (1973) Selenium: Biochemical role as a component of glutathione peroxidase. Science 197: 588–590.
Singh NP, McKoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of damage in individual cells. Exp Cell Res 175: 184–191.
Spetrie H, Klassen TW, Klassen PS, O'Dell JR, Kay HD (1989) Selenium and immune response 2: enhancement of murine cytotoxic T lymphocyte and natural killer cytotoxicity *in vivo*. J Leuk Biol 45: 215–226.
Tan DX, Reiter RJ, Chen LD, Poeggeler B, Manchester LC, Barlowwalden LR (1994) Both physiological and pharmacological levels of melatonin reduce DNA adduct formation induced by the carcinogen safrole. Carcinogenesis 15: 215–218.
Zhao CY, Shi YM, Wang WF, Lin WZ, Fan BT, Jia ZJ, Yao SD, Zheng RL (2002) Fast repair activities of quercetin and rutin toward dGMP hydroxyl radical adducts. Radiat Phys Chem 63: 137–142
Zhao J, Liu XJ, Ma JW, Zheng RL (2004) DNA damage in healthy term neonate. Early Hum Dev 77: 89–98.
Zhou YC, Zheng RL (1991) Phenolic compounds and an analog as superoxide anion scavengers and antioxidants. Biochem Pharmacol 42: 1177–1179.