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Melatonin reduces chromosomal breakage and DNA damage in Down syndrome blood lymphocytes

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Abstract A possible protective effect of melatonin on chromosomal damage induction was evaluated in lymphocytes from patients with Down syndrome and normal control. This included determination of chromosomal breakage induced by mitomycin C as H_2O_2 promoter in lymphocyte cultured with and without different concentrations of melatonin. Comet assay was also used to detection of oxidative stress dependant DNA damage in Down syndrome children patient's lymphocytes. Melatonin treatment significantly decreased chromosomal breakage and DNA damage in DS lymphocytes. These results suggested that high rate of chromosomal breakage and DNA damage in DS lymphocytes may be related to the increase in oxidative stress damage reported in these patients. Melatonin reduces chromosomal and DNA damage in DS lymphocytes These observations may have implications for human protection against damage due to endogenously produced free radicals and also due to exposure to free radical producing physical and chemical mutagens. In conclusion supplementation of melatonin could play an important role in preventing, treating or at least delaying the progression of DS complications, particularly compensating for the decreased level of melatonin.

Introduction

Down syndrome (DS) is a gene related disease emerging from the presence of an extra copy of chromosome 21 (Trisomy 21) and this disease estimated to affect 1 out of 750 live births (Zitnanova *et al.*, 2006).DS related pathological features are a direct result of excessive expression of chromosome 21 allocated gene of superoxide dismutase (SOD). Children with DS are reported to have increased activity of the antioxidant enzyme SOD that can cause oxidative damage to cell by increasing hydrogen peroxide level (Zana *et al.*, 2007). Oxidative stress in a phase of developmental growth may injure many fetal organs and tissues such as neurodegenration and its related pathology of dementia and early AD onset.

Melatonin (N-acetyl-5-methoxytryptamine) is a powerful antioxidant that is mainly secreted from pineal gland. The down regulation of melatonin endogenous levels per day is a hallmark of DS patients' blood samples (Uberos *et al.*, 2010). Melatonin is a potent antioxidant ceasing cell oxidative stress not only by directly scavenging free radicals but also indirectly through modulating the

expression of antioxidant enzymes and as a counteracting molecule against oxidative stress-induced DNA damage (Tan *et al.*, 2000). These triple mechanisms of action render melatonin literally a 'super-potent' antioxidant against DS dependent-superoxide dismutase induced damage on all relevant levels (Garcia *et a.*, 2014).

Recently published articles show that melatonin plays a protective role in both initial and advanced stages of diseases whose pathogenesis involves damage by oxygen free radicals (Reiter *et al.*, 2014). Therefore, it is anticipated that the melatonin may be an appropriate approach to reduce chromosomal damage in DS children in order to control its related pathological features.

Materials and Method

Patients

The strategy of this study is to *in vitro* investigate the effect of melatonin on diminishing the progression of DS accompanied syndromes such as cancer and neurodegenerative diseases which appears mainly as a function of oxidative stress induced chromosomal breakage. In order to do that, the study subjects were divided into two groups; (Group1) DS children patients and (Group2) normal children. Each group consisted of a fifteen children who were presented as out patients' to the genetic unit, children hospital, Mansoura University. Their ages ranged from neonates months to 4 months.

Isolation of plasma

Blood samples were obtained from the antecubital vein in chilled tubes containing heparin as anticoagulant. The blood samples were centrifuged at 1500 g for 10 min toseparate blood cells from plasma. The plasma was kept frozen at -80 $^{\circ}$C until it is used for determination of biochemical parameters.

Chromosomal culture

Chromosomal study was carried out according to the method of Rooney and Czepulkowski (1997). Half ml of the lymphocyte-rich plasma was introduced into a sterile 15 ml culture tube containing 5 ml of mixed culture medium, supplemented with 0.2 ml (0.048 mg) phytohemaglutinin, to initiate mitosis. Afterwards, the contents of each tube were mixed gently. Then, the culture tube was incubated for 72 h at 37 °C in a horizontal position. The horizontally position allows the cells to settle over a large area of the culture tube, which provides optimal culture conditions for cell growth and proliferation. After 70 hours of incubation 0.05 ml of colcemide solution (10 μg /ml) was added to each culture tube and mixed by shaking the tube gently. The culture tubes were returned to the incubator for 1.5 hours. The culture tubes were centrifuged at 1000 rpm for 10 minutes. The cell pellet was incubated at 37°C for 15 min with hypotonic solution (KCL, 0.4%). The supernatant fluid was removed and the sediment was resuspended in a small amount of the fresh fixative [methanol: glacial acetic acid (3:1)] enough to make the solution slightly turbid. On a pre-cleaned and grease free slide, 2-3 drops of the suspension were placed, sufficiently far apart so that they do not meet after spreading. The slide was dried by putting it on 60 °C hotplate for 2 minutes. The slide was stained for 6 minutes in freshly prepared Giemsa solution.

The effect of melatonin on chromosomal breakage was investigated by adding melatonin at concentrations of 0.16, 0.48 & 0.8mg/ml in presence of mitomycin C (16 mg/ml), H_2O_2 promotor, to the culture. The cultures were incubated at 37^{o} C for 72

Isolation of peripheral blood lymphocytes for comet assay

Lymphocytes were immediately prepared from two ml freshly drawn, heparin-anticoagulated whole venous blood with Histopaque 1077 (Sigma, Germany). The interface was collected after centrifugation at 200g for 15 min at room temperature. The cells were then washed in phosphate-buffered saline (PBS) (pH 7.2) (200g, 5 min). The order of the yields was $10x^{6}$ cells/ml blood.

Comet assay (Single cell gel electrophoresis)

Evaluation of DNA damage using single-cell gel electrophoresis (Comet Assay) was performed after different treatments of cultured lymphocytes according to (Singh *et al.* 1988) One hundred cells were analyzed on each slide using the comet assay II automatic digital analysis system (Nandhakumar *et al.* 2011).

Method of comet assay

The comet assay is suitable for the quantification of genomic damage at the individual cell level. The samples were homogenized in chilled homogenizer buffer, pH 7.5 containing 75mM NaCl and 24mM Na2EDTA pH 13 to obtain a 10% tissue solution. A potter type homogenizer was used and samples were kept on ice during and after homogenization. Six microliters of the homogenate were suspended on 0.5% low melting agarose and sandwiched between a layer of 0.6 % normal-melting agarose and a top layer of 0.5 % low melting agarose on fully frosted slides. The slides were kept on ice during the polymerization of each gel layer. After the solidification of the 0.6 % agarose layer, the slides were immersed in a lyses solution (1% sodium surcosinate, 2.5 mNaCl, 100mM Na2EDTA, 10mm Tris-HCl, 1% tritonX-100 and 10% DMSO) at 4^{o}C. After 1 h, The slides were placed in electrophoresis buffer (0.3M NaOH, 1 mM Na2EDTA, Ph 13) for 10 minutes at 0oC to allow DNA to unwined. Electrophoresis was performed for 10 min at 300mA and 1V/cm. The slides were neutralized with tris–Hcl buffer, pH 7.5, and stained with 20µg/ml ethidium-bromide. Each slide was analyzed using the Leitz Orthoplan (Wetzlar, Germany) epifluorescence microscope. One hundred cells were analyzed on each slide using the comet assay II automatic digital analysis system. Perspective tail length (µm) is the distance of DNA migration from the center of the body of the nuclear core and is used to evaluate the of DNA damage. The tail moment is defined as the product of the tail length and the fraction of total DNA in the tail (Tail moment =tail length x % of DNA in the tail). Both tail length and tail intensity are measured automatically by image analysis software (Sasaki *et al.* 1997; Robbiano *et al.* 2004).

Results

Karyotyping of Down syndrome patients:

The human chromosomes, in a lymphocyte arrested in metaphase, of down syndrome patients show three copies of chromosome 21 as illustrated in fig. (A&B).

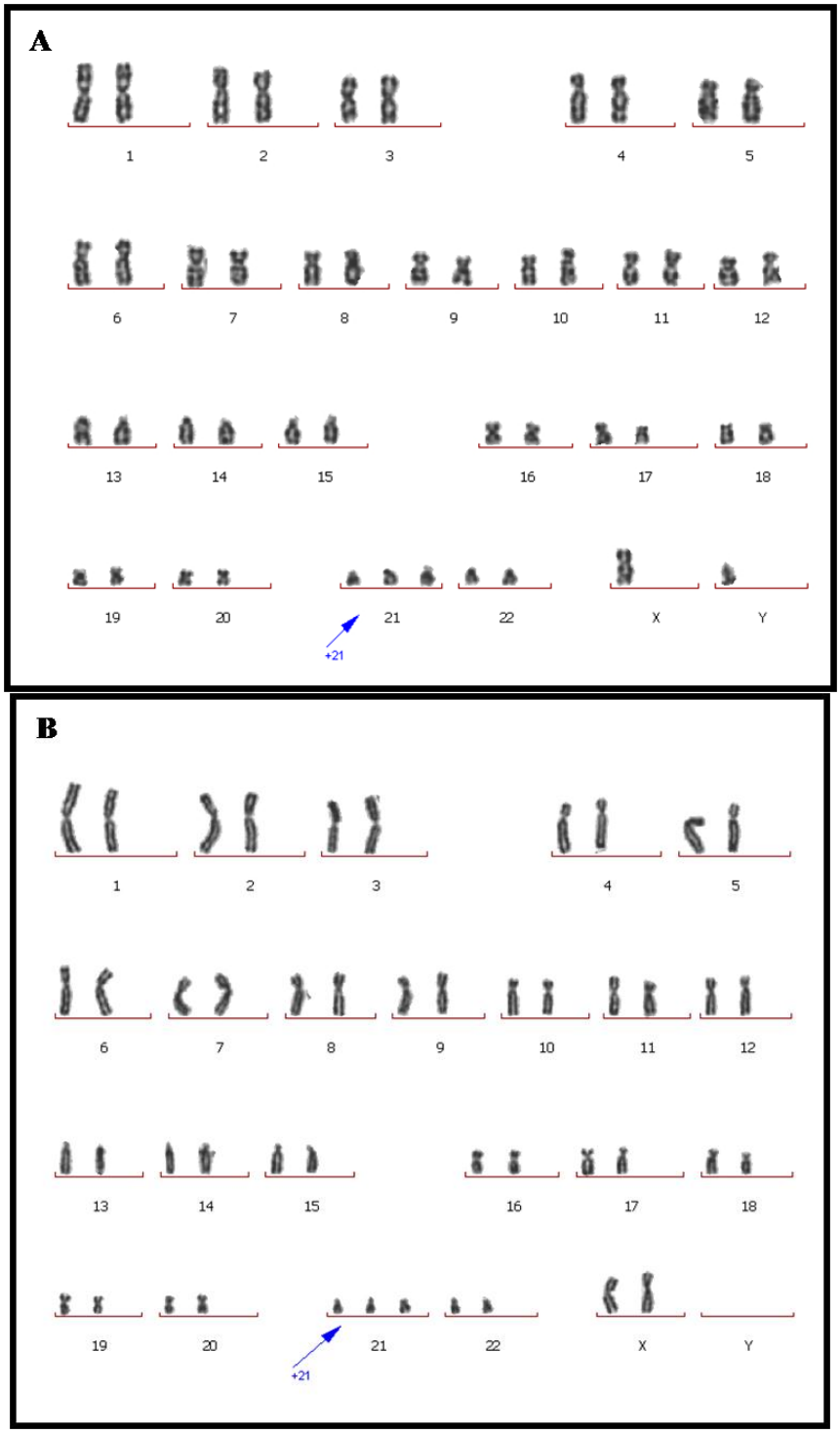

Fig . (1A&B): Karyotype of male (A) and female (B) down syndrome children patient, the presented data show the presence of an extra copy of chromosome 21.

Effect of different concentration of melatonin on oxidative stress induced chromosomal damage:

This part of the study was conducted *in vitro* in order to develop a dose response curve showing the effective dose of melatonin against the destructive effect of mitomcyin C (MMC), H_2O_2 promoter, on induction of chromosomal damage.In order to do that, lymphocytes of Down syndrome children patients at the same age were collected and cultured in the presence of mmc (16 mg/ml). In addition, different concentrations of melatonin (0.16, 0.48 & 0.8mg/ml) were added to the culture medium along with the MMC. The resulting data showed that MMC induced a significant increase at number of damaged chromosomes in Down syndrome patient's lymphocytes. The

concomitant treatment of lymphocytes with different concentrations of melatonin resulted in a significant concentration related protection against MMC induced oxidative chromosomal damage (Fig. 2).

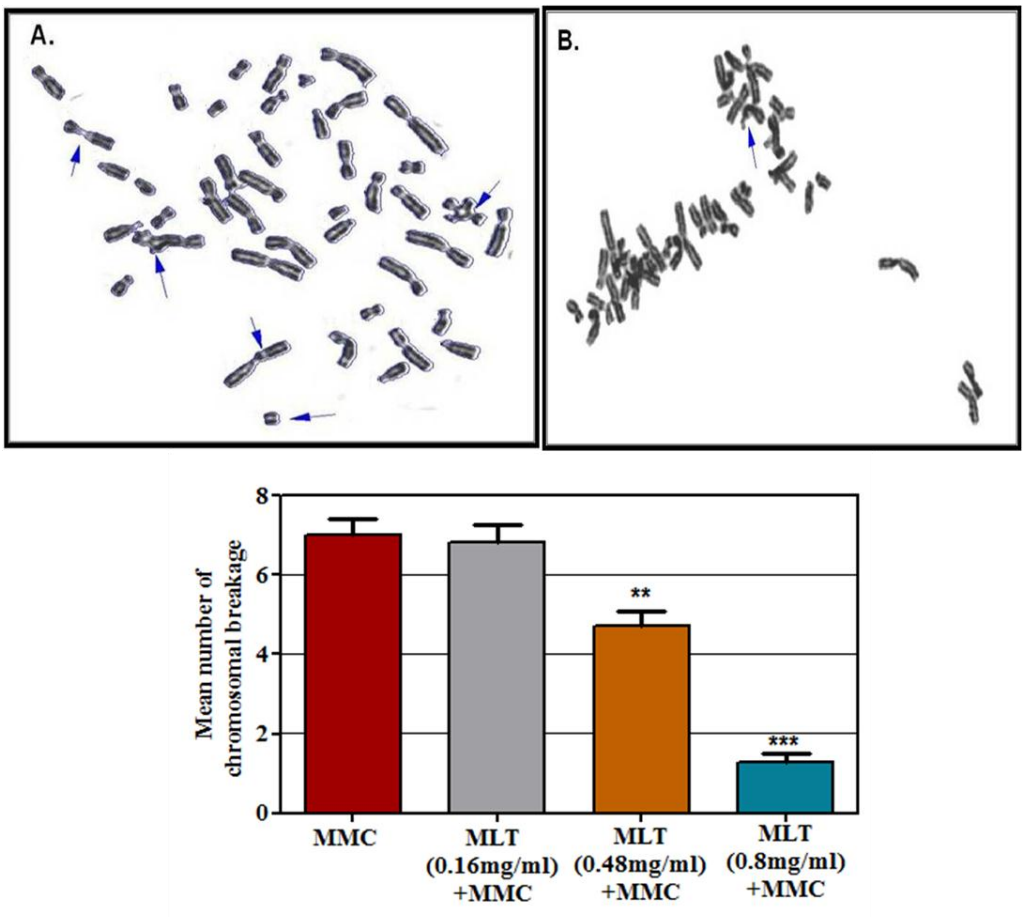

Fig.2: The protective effect of different concentrations of melatonin against the MMC dependant chromosomal damage at Down syndrome patients' lymphocytes.

(A, B) Karyotyping of down syndrome patients' lymphocyte showing the protective effect of 0.8mg/ml melatonin against chromosomal damage induced by MMC in vitro; **(A)** Melatonin untreated down syndrome patient's lymphocytes show multiple of damaged chromosomes (blue arrows)**; (B)** Melatonin treated lymphocytes show a few number of chromosomal damaged (blue arrow).The change of the number of chromosomal breakage at each group was compared to the melatonin free group. The presented data shows a dose dependant protective effect of melatonin against chromosomal damage, with a highly significant cytoprotective effect of melatonin at concentration of 0.8mg/ml.

Detection of oxidative stress dependant DNA damage in Down syndrome children patient's lymphocytes

Lymphocyte cells of Down syndrome young children patients were tested using comet assay approach for the percentage of DNA damage in (a) mmc untreated lymphocytes, (b) MMC treated lymphocytes and (C) MMC+MLT treated lymphocytes. The percentage of DNA damage was determined by the percentage of DNA concentration and tail length of the migrated cells. The presented data revealed a significant decrease in tail length of fragmented DNA at MMC+MLT treated group in comparison to MMC only treated lymphocytes, as shown in fig. 3.

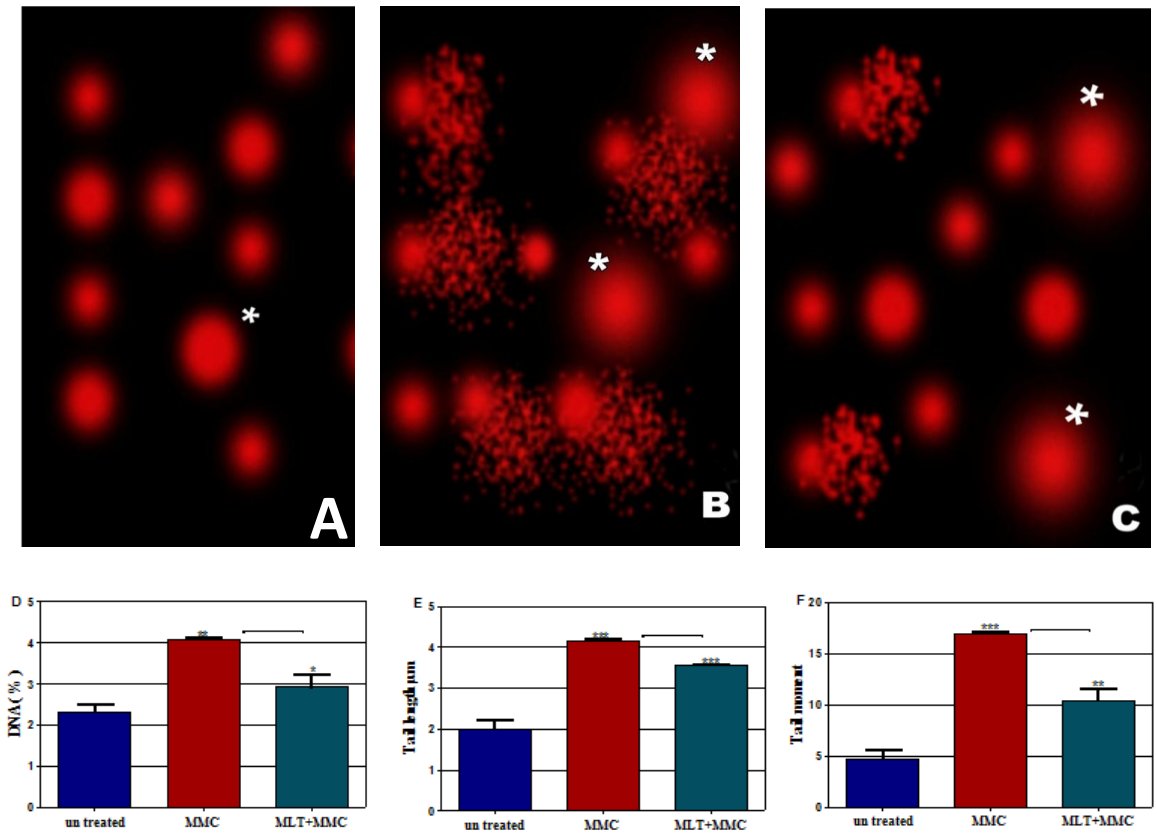

Fig .3: A-C: photomicrographs of comet assay of lymphocyte cells of Down syndrome patients' during MLT treatment. (A) Untreated Down syndrome cells, (b) MMC treated cells and (c) MMC+ MLT treated cells. White stars symbol to damaged DNA. Fig D-F: Lymphocyte cells treated with melatonin, (D) DNA concentration, (E) tail length of lymphocyte cells (F) Tail moment of lymphocyte cells.

Discussion

Children with Down syndrome (DS) have increased vulnerability to oxidative stress caused by overexpression of superoxide dismutase (SOD), an antioxidant enzyme coded on chromosome 21. The presence of an extra-copy of SOD gene on the distal region of chromosome 21(21q22 band) is suggested as the main cause for the oxidative stress related clinical features in DS patients (Schwaiger *et al.,* 1989 andPincheira *et al.,*1999).

The increased oxidative stress may lead to oxidative damage of important macromolecules including DNA and proteins (Zitnanova *et al.*, 2006) leading to DS associated complications. The levels of 8-hydroxy-2-deoxyguanosine a biomarker ofoxidative damage to DNA, and malondialdehyde a biomarker of lipid peroxidation, are significantly elevated in individuals with DS(Jovanovic *et al.,* 1998).Dysregulated production of ROS during oxidative stress can result in DNA modifications, including base alterations, single (SSBs) and double strand breaks (DSBs), sister chromatid exchanges (SCEs) and DNA-protein crosslinks (Hwa Lee 2013). In line with these observations the present results demonstrated a significant susceptibility of chromosomal breakage towards MMC (H_2O_2 promoters) in lymphocytes of DS children. This was confirmed by the significant increase in the tail length indicating DNA damage in DS cultured lymphocytes subjected to MMC compared to the normal children. Our results are in agreement with those recently obtained using the comet assay that children with DS displayed an increased sensitivity to hydrogen peroxide in contrary to adults with enhanced amounts of oxidized purines in DS lymphocytes (Zana *et al.* 2006 and Subramaniam *et al.,* 2013) supporting the existence of elevated oxidative stress and that trisomic cells are more vulnerable to oxidative stress (Muchova *et al.* 2001; Sanij *et al.* 2001).

On the basis of the foregoing, it is anticipated that one of the leading theories on the pathogenesis and complications associated with

DS is that ROS have an early role in DNA damage and cell death. Therefore, the possibility of the therapeutic use of antioxidants seems to be rational, reducing the incidence and severity of disease (Sulthana *et al.*, 2012). Supplementation of exogenous antioxidants could play an important role in preventing, treating or at least delaying the progression of DS complications, particularly compensating for the decreased level of endogenous antioxidants. Moreover, the manipulation of endogenous cellular defense mechanisms via endogenous antioxidants may comprise an innovative approach to therapeutic (Srinivasan *et al.*, 2005; Pandi-Perumal *et al.*, 2013).

Melatonin is also known as a powerful antioxidant that acts either directly by scavenging ROS such as H_2O_2, NO superoxide anion and hydroxyl radicals (Poeggeler *et al.*, 2002) or indirectly by modulating the activity of the antioxidant enzymes such as SOD, GPx and GT and GSH (Fischer *et al.*, 2013).

In the present study melatonin decreased the susceptibility of chromosomal breakage and DNA damage of lymphocytes of DS patients. The efficacy of melatonin in preventing oxidative damage in either cultured neuronal cells or in the brains of animals treated with various neurotoxic agents, suggests that melatonin has a potential therapeutic value as a neuroprotective drug in treatment of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, stroke, and brain trauma (Pandi-Perumal *et al.*, 2013).

The protective role of melatonin in DNA damage response observed in the present study is in agreement with previous findings (Tan *et al.* 1998 ;Vijayalaxmi *et al.*, 1998a ; Vijayalaxmi *et al.* 1998b). Because melatonin is known free radical scavenger (Tan *et al.*, 2002), many studies have suggested that melatonin may protect DNA against free radical damage by scavenging ROS including •OH and H_2O_2 (Tan *et al.*, 2000). In addition to stimulating DNA repair capacity, melatonin may aid in inactivating the DNA-damaging agent (Liu *et al.*, 2013). Our results lend credence to these findings and demonstrate for the first time that melatonin may also protect DNA repair capacity against chromatid break formation caused by the DNA damaging agent MMC *in vitro*, as measured by the comet assay. MMC induced chromatid break formation involving inability to complete DNA replication past cross-links and recombination events occurring at the sitesof blocks to replication (Sognier & Hittelman., 1986). In a previous study, melatonin can have a protective effect against oxidative DNA damage by chemical inactivation of a DNA-damaging H_2O_2 as well as by stimulating DNA repair (Sliwinski *et al.*, 2007).

In conclusion our observations in Down syndrome blood lymphocytes treated with melatonin in vitro are important with regard to free radical scavenging and genome protection. Since melatonin is not foreign to the human body, these results should provide impetus for further research leading to the use of melatonin, and the biological activity of this endogenously synthesized pineal hormone, in protection of the genome from natural and other DNA damaging agents.

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الميلاتونين يقلل تكسر الكروموسومات وتلف الحمض النووى فى الخلايا الليمفاويه للمرضى أطفال متلازمة داون

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تم تقييم التأثيرالوقائي المحتمل للميلاتونين على تكسيرالكروموسومات في الخلايا الليمفاوية للمرضى الأطفال الذين يعانون من متلازمة داون والأطفال الأصحاء. وهذا يشمل تحديد تكسير الكروموسومات الناجم عن اضافة الميتوميسين سى (MMC) كعامل مستحث لانتاج فوق اكسيد الهيدروجين(H_2O_2) في مزرعة الخلايا الليمفاويه فى وجود تركيزات مختلفه من الميلاتونين. كما استخدم اختبار المذنب (comet assay) للكشف عن مدى تحطم الحمض النووي على مستوى الخليه. وعند العلاج بالميلاتونين انخفضت نسبة تكسيرالكروموسومات والاضرار فى الحمض النووي بشكل ملحوظ في الخلايا الليمفاوية لمرضى المتلازمه. وتشيرهذه النتائج إلى أن الزيادة في تكسير الكروموسومات و تلف الحمض النووي في الخلايا الليمفاوية قد تكون ذات صلة إلى زيادة الضررالناتج عن الإجهاد التأكسدي التي وجدت في هؤلاء المرضى. ويرجع تأثيرالميلاتونين لتقليل تكسير الكروموسومات و تلف الحمض النووي في الخلايا الليمفاوية الى تقليل الأضرارالناجمة عن الجذور الحرة أو التعرض للجذور الحرة نتيجةالنشاط الخلوى. و يمكن أن يلعب الميلاتونين كمكمل غذائى دورا هاما فيمنع وعلاج أو على الأقل تأخيرتطورمضاعفات مرض متلازمة داون، ولا سيما التعويض عن انخفاض مستوى الميلاتونين.