Food and Chemical Toxicology 50 (2012) 583–589

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

# Fisetin prevents fluoride- and dexamethasone-induced oxidative damage in osteoblast and hippocampal cells

Iwona Inkielewicz-Stepniak [a,*], Marek W. Radomski [b], Michal Wozniak [a]

[a] Department of Medical Chemistry, Medical University of Gdansk, Poland
[b] School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Ireland

ARTICLE INFO

*Article history:*
Received 6 September 2011
Accepted 10 December 2011
Available online 17 December 2011

*Keywords:*
Fluoride and dexamethasone
Osteoblast cells
Hippocampal cells
Oxidative/nitrosative stress
Apoptosis
Fisetin

ABSTRACT

Fluoride intoxication and dexamethasone treatment produce deleterious effects in bone and brain. The aim of this study was to evaluate the effect of fluoride (F) and dexamethasone (Dex) co-exposure on oxidative stress and apoptosis in osteoblast-like MC3T3-E1 and hippocampal HT22 cell lines. Co-exposure to F and Dex resulted in a concentration-dependent decrease in cell viability, induction of apoptosis and increased generation of reactive oxygen species (ROS) and nitric oxide (NO) following 72 h of incubation. Fluoride-induced apoptosis in MC3T3-E1 and HT22 cells was attenuated by catalase and L-NNMA, indicating a role for $H_2O_2$ and NO as mediators of cytotoxicity. Dexamethasone-induced apoptosis was associated with $H_2O_2$ generation in both cell lines and it was attenuated during co-incubation with catalase. These data indicate that co-exposure to F and Dex amplifies their respective cytotoxicity in $H_2O_2$- and NO-dependent manner. As flavonoid fisetin prevented F- and Dex-induced cytotoxicity the potential role of this product in pharmacology and diet may be considered.



## 1. Introduction

Humans are normally exposed to several chemicals at the same time rather than to an individual chemical. Medical treatment and environmental exposure generally consists of multiple exposures. A xenobiotic interaction may increase or decrease the toxic effects of one or many substances (Inkielewicz-Stepniak and Czarnowski, 2010; Mittal and Flora, 2007). Particularly important are interactions between such toxicants to which exposure is the most common. Examples of such substances are fluoride (F) and dexamethasone (Dex). Fluoride exposure is a serious public health problem in many parts of the world where drinking water contains more than 1 ppm of fluoride (Krishnamachari, 1986; WHO, 2002). Excessive intake of F may lead to pathological changes in teeth and bones e.g. dental and skeletal fluorosis. It is also known that F passes the blood–brain barrier and damages the hippocampus (Inkielewicz-Stepniak and Czarnowski, 2010; Lu et al., 2000). Interestingly, high levels of F in drinking water (3–11 ppm) affect the central nervous system directly without first causing the physical deformations of skeletal system. Fluorosis may be caused by occupational exposure or by excessive intake of F from drinking water and from food. Many studies have shown decreased intelligence in children from F endemic areas (Lu et al., 2000; WHO, 2002).

Corticosteroids, especially Dex, are associated with increased risk of osteoporosis in 50% of patients on long-term corticosteroid therapy (Gourlay et al., 2007). In the presence of corticosteroids, calcium absorption and osteoblast activity decrease. The net results are a negative calcium balance, increased bone breakdown and ensuing bone loss (Conradie et al., 2007; Gourlay et al., 2007). Also hippocampus is one of the principal targets for glucocorticoid (GC) action in the brain (Analía et al., 2008; McEwen et al., 1999; Sapolsky, 1990; You et al., 2009). Many studies have indicated that either F or Dex induces free radical toxicity in humans and animals (Analía et al., 2008; Inkielewicz-Stepniak and Czarnowski, 2010; Mittal and Flora, 2007; Tome and Briehl, 2001; WHO, 2002; You et al., 2009). But the effects of co-exposure with F and Dex have not been elucidated yet. Because fluoride is a ubiquitous environmental element and dexamethasone is a commonly used corticosteroid we decided to investigate the effect of co-treatment with both substances as a result of uncontrolled exposition. Interestingly, Rich and Ensinck (1961) introduced sodium fluoride for the treatment of osteoporosis, also induced by glucocorticoids. There was a controversy about using NaF in this treatment as many physicians doubted its efficacy and have been concerned by the frequency of side effects, particularly the risk of increased bone fragility. Therefore, sodium fluoride is not recommended for clinical use in glucocorticoid-induced osteoporosis as a first or second line therapy (Boulos et al., 2000; Cohen and Adachi, 2004).

Considering all the information, it was of interest to investigate the contribution of nitrosative and oxidative stress to apoptosis in

* Corresponding author. Address: Department of Medical Chemistry, Medical University, Gdansk, Debinki St., 80-211 Gdansk, Poland.
*E-mail address:* iinkiel@gumed.edu.pl (I. Inkielewicz-Stepniak).

0278-6915/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2011.12.015

osteoblast-like MC3T3-E1 and hippocampal neuronal HT22 cells co-exposed to F and Dex. Oxidative and nitrosative stress is widely recognized as an important mediator of apoptosis in different cells and plays a pivotal role in the pathogenesis of several diseases. Epidemiologic data have indicated that certain dietary additives can provide an effective defense against oxidative stress, thus have a potential in the treatment of a variety of diseases (Kimira et al., 1998; Sengupta, 2004). Flavonoids are a class of natural biological products that have evolved to protect plants from the oxidative damage. Fisetin (3,3′,4′,7-tetrahydroxyflavone) is a flavonoid found in fruits, vegetables, nuts and wine at concentrations of 2–160 μg/g with an average daily intake estimate of 0.4 mg (Kimira et al., 1998; Park et al., 2007; Sengupta, 2004; Woodman and Chan, 2004). Fisetin is also added to nutritional supplements at very high concentrations and has a variety of pharmacological effects including antioxidant and anti-inflammatory activity acting mainly as a free radical scavenger (Kimira et al., 1998; Park et al., 2007; Sengupta, 2004; Woodman and Chan, 2004).

The purpose of the present study was to measure the pharmacological interactions between F and Dex on apoptosis, reactive oxygen species generation and nitric oxide levels in osteoblast and hippocampal cell lines. We have also investigated if F- and Dex-induced nitrosative stress and apoptosis could be prevented by fisetin, a flavonoid with high equivalent antioxidative capacity.

## 2. Materials and methods

### 2.1. Chemicals

The concentrations of fluoride and dexamethasone used in these experiments were carefully chosen according to literature data for *in vitro* experiments (Chua et al., 2003; Conradie et al., 2007; Ozaki et al., 2010; Roma et al., 2009; Wang et al., 2011; Yan et al., 2009).

Dexamethasone solution was prepared by diluting dexaven (*Jelfa SA*, *Poland*, CAS: 50-02-2) in sterile water, while the fluoride solution was obtained from natrium fluoride (*POCH*, *Poland*, CAS: 7681-49-4) dissolved in dimethyl sulfoxide (DMSO). Fisetin (*Sigma Aldrich*, *Poland*, CAS: 528-48-3) was dissolved in ethanol. N5-[imino(methylamino)methyl]-L-ornithine, citrate (L-NMMA) and catalase (*Sigma Aldrich*, *Poland*) were dissolved in sterile water.

### 2.2. Cell culture

Hippocampal neuronal HT22 cells and osteoblast-like MC3T3-E1 cells were obtained from American Type Culture Collection (ATCC). HT22 cell line were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin. MC3T3-E1 cell line was cultured in alpha minimum essential medium supplemented with 10% fetal bovine serum 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$. Cells were regularly split and subcultured up to ~80–90% confluence before experimental procedures.

### 2.3. Treatments

MC3T3-E1 and HT22 cell lines were treated with F (100, 250 μM – 1.9 and 4.75 ppm, respectively) and/or Dex (100, 250 nM) for 72 h. Catalase (400 units/ml), L-NNMA (1 mM) or fisetin (60 μM) was added 1 h prior to the treatment with higher concentration of F and/or Dex. Working solutions were prepared in appropriate serum-free medium *ex tempore* every time before adding to the cell line. Vehicle controls were performed using DMSO and ethanol at the final concentration of 0.1%.

### 2.4. Measurement of cell viability

The MTT assay, which measures cell proliferation and cytotoxicity, was used to verify the viability of MC3T3-E1 and HT22 cell lines. The cells were seeded into 96-well plates at a concentration of $1 \times 10^4$ cells/well (MC3T3-E1) and $5 \times 10^3$ cells/well (HT22). 24 h after seeding, cell were treated as specified in Section 2.3. Subsequently, after treatment the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution for 4 h at 37 °C, and the solution was then replaced with DMSO. The absorbance was measured at 570 nm by a microplate reader (FLUOstar OPTIMA, BMG Labtech). Cell viability was expressed as percentage of MTT reduction.

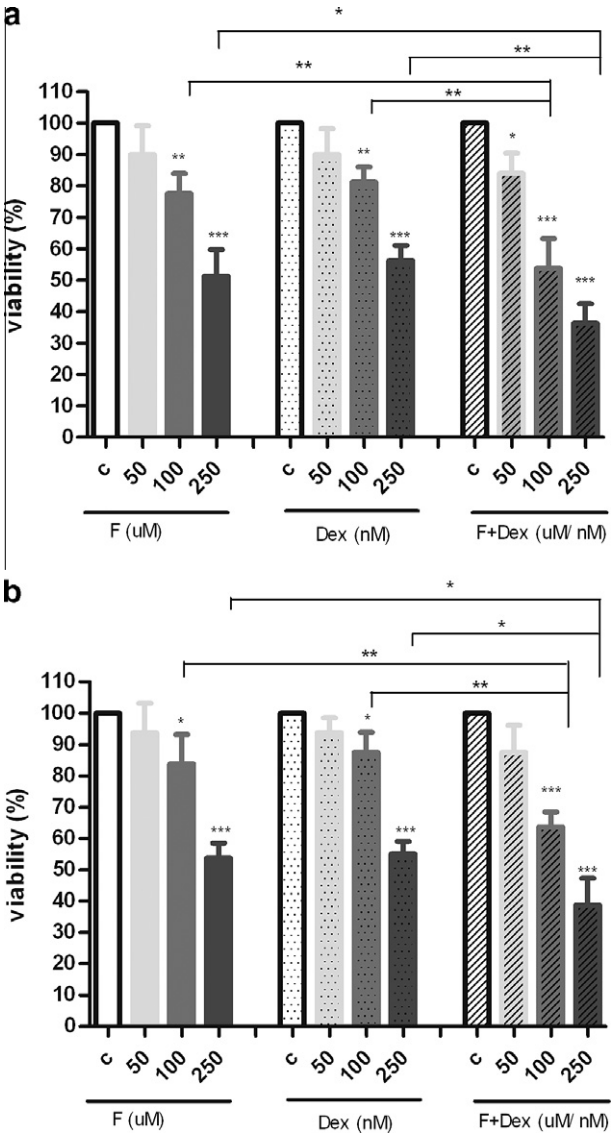


**Fig. 1.** Reduction of cell viability by F, Dex and F + Dex. Effects of 72 h treatment with F, Dex and F + Dex on MC3T3-E1 (a) and HT22 (b) cell viability. Data are means ± SD of 3–4 separate determinations. $^{*}p < 0.05$; $^{**}p < 0.01$; compared with control or as indicated.

### 2.5. Detection of ROS

ROS generation was measured using flow cytometry. Briefly, the cells were seeded into 12-well plates at a concentration of $1 \times 10^6$ cells/well (MC3T3-E1) and $1 \times 10^5$ cells/well (HT22). 24 h after seeding, cells were treated as specified in Section 2.3, then collected and incubated with 10 μM of 2,7-dichlorofluorescein diacetate (DCF-DA) for 30 min at 37 °C in a humidified atmosphere containing 5% $CO_2$/95% air to assess ROS-mediated oxidation of DCF-DA to the fluorescent compound 2,7-dichlorofluorescin DCF (24). Fluorescence of oxidized DCF was measured at an excitation wavelength of 480 nm and an emission wavelength of 525 nm using a flow cytometer (BD FACSArray; UK).

### 2.6. Determination of total nitric oxide level (NO)

Nitric oxide levels were measured using the colorimetric assay (Stressgen Diagnostic Kit). The obtained results represented the sum of nitrite and nitrate levels. All nitrates in a sample were initially converted enzymatically into nitrites by nitrate reductase and then transformed in the Griess reaction into an azo dye allowing for colorimetric detection ($\lambda = 540$ nm). Briefly, the cells were seeded into 12-well plates at a concentration of $1 \times 10^6$ cells/well (MC3T3-E1) and $1 \times 10^5$ cells/well (HT22). 24 h after seeding, cells were treated as specified in Section 2.3, then the cell culture supernatants were mixed with equal volume of the Griess reagent

(1% sulfanilamide and 0.1% naphthylethylene-diamine in 5% phosphoric acid). The absorbance was measured at 540 nm and NO concentrations were determined using a standard curve of sodium nitrite generated by known concentrations.

### 2.7. Flow cytometric analysis of apoptosis

The Annexin V-FITC apoptosis detection kit (BD Pharmingen) was used to detect apoptosis according to the manufacturer's instructions. Briefly, cells MC3T3-E1 ($5 \times 10^6$) and HT22 ($1 \times 10^6$) were seeded into 25-cm$^2$ tissue culture flasks. 48 h after seeding, cells were treated as specified in Section 2.3, then collected and washed twice with cold PBS. 5 μl of Annexin V and 2.5 μl of PI were added to the cells, which were resuspended in binding buffer. 5 μl of Annexin V and 2.5 μl of PI were added to the cells, which were resuspended in binding buffer. The cells were gently shaken while incubating for 15 min at room temperature in the dark, then cells were analyzed by flow cytometry (BD FACSArray; UK) within 1 h. Annexin V labeled with a fluorophore enabled to identify cells in the early stage of apoptosis, and PI being a fluorescent nucleic acid binding dye, was meant for staining cells in the medium and late stages of apoptosis. A total of 20,000 events were acquired for each sample. Analysis was based on gating a subpopulation of cells by forward scatter versus side scatter. The intermediate to large cell population was the gated region used to calculate the apoptotic rate. The apoptotic rate was calculated as the percentage of Annexin V-positive and PI-negative cells divided by the total number of cells in the gated region.

### 2.8. Statistical analysis

The experimental results were expressed as the mean ± SD for triplicates determination of 3–4 separate experiments. The results were analyzed by using Tukey's test and one-way ANOVA. $p$ values <0.05 was considered statistically significant.

## 3. Results and discussion

Dexamethasone is one of the most commonly used corticosteroids in therapeutics (Vyvey, 2010; WHO, 2003). Fluoride is an important environmental toxicant, which has natural and industrial sources (Lu et al., 2000; WHO, 2002). Therefore, humans can be simultaneously exposed to F and Dex. Both xenobiotics exert adverse effect on bone and brain (mainly hippocampus) (Analía et al., 2008; Conradie et al., 2007; Gourlay et al., 2007;Inkielewicz-Stepniak and Czarnowski, 2010; Krishnamachari, 1986; Lu et al., 2000; McEwen et al., 1999; Sapolsky, 1990; WHO, 2002; You et al., 2009). In the present study, for the first time, we have investigated the effect of co-exposition to fluoride and dexamethasone on apoptosis, reactive oxygen species generation (ROS), and nitric oxide (NO) levels in osteoblast-like and hippocampal cell lines. In addition, we have studied the effects of fisetin – a dietary cytoprotective flavonoid on F- and Dex-induced cellular damage.

First, we investigated the effects of F, Dex and F + Dex on MC3T3-E1 and HT22 cell viability (Fig. 1). Incubations with F, Dex and F + Dex for 72 h resulted in decreased MC3T3-E1 and HT22 cell viability. Co-exposition to both xenobiotics enhanced significantly ($p < 0.05$; $p < 0.01$) cell cytotoxicity when compared to F or Dex alone.

Interestingly Wang et al. (2011) reported decreased osteoblast viability as early as following 12 h of exposure to F.

We have also found that the loss of cell viability was reversed in a concentration-dependent manner by fisetin and this effect was maximal at 60 μM. Fisetin is a natural flavonoid from fruits and vegetables that exhibits antioxidant, neurotrophic, anti-inflammatory, and anti-cancer effects in various disease models (Khan et al., 2008; Kimira et al., 1998; Park et al., 2007; Sengupta, 2004; Woodman and Chan, 2004). Fig. 2a and b shows that fisetin (20–80 μM) when incubated for 72 h protected against cell death caused by F (250 μM), Dex (250 nM) – (data are not shown) and F + Dex (250 μM + 250 nM) in MC3T3-E1 and HT22 (Fig. 2). The 60 μM of fisetin was then used in subsequent experiments.

We have also found that co-exposure to F and Dex leads to apoptosis in osteoblast and hippocampal cells. Fig. 3 shows the pro-apoptotic effect of F-, Dex- and F + Dex- in MC3T3-E1 and HT22 cells. Co-exposure to F and Dex resulted in a significant increase

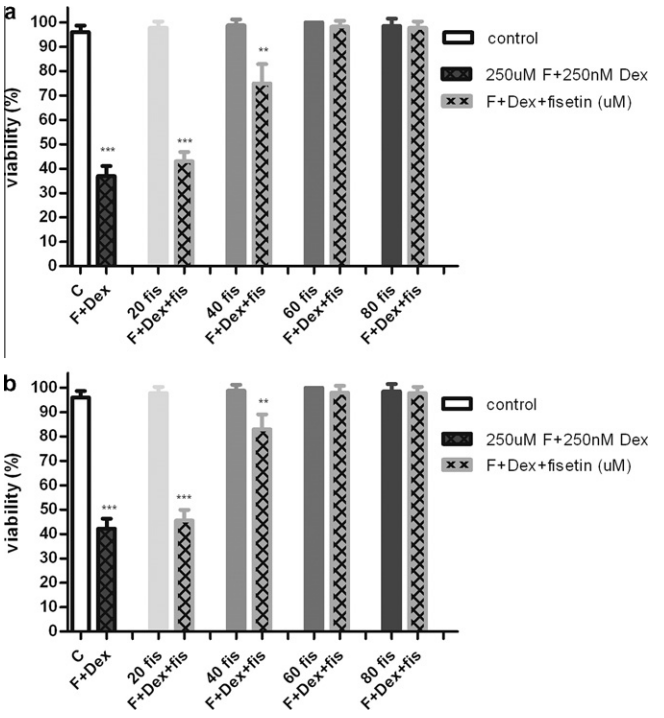


**Fig. 2.** Reversal of F- and Dex-induced cytotoxicity by fisetin. Effects of increasing concentrations of fisetin on F + Dex-induced cytotoxicity in MC3T3-E1 (a) and HT22 (b). Data are means ± SD of 3–4 separate determinations. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ F + Dex-treated cells v/s F + Dex-treated cells in the presence of fisetin.

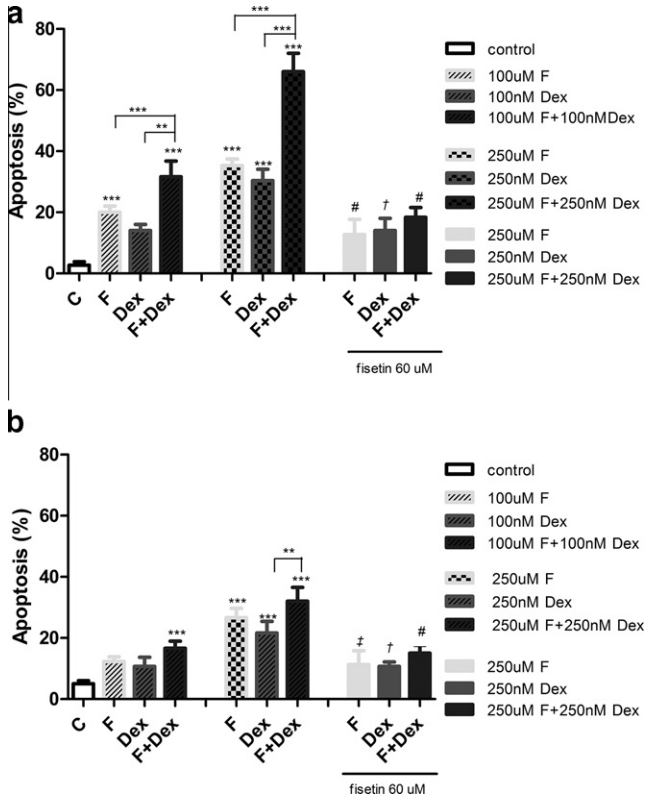


**Fig. 3.** Increase in apoptosis in MC3T3-E1 and HT22 cells following exposure to F, Dex, F + Dex and protective effect of fisetin. Fisetin inhibits F-, Dex-, F + Dex-induced apoptosis in MC3T3-E1 (a) and HT22 (b) cells. Data are means ± SD of 3–4 separate determinations. $^{**}p < 0.01$; $^{***}p < 0.001$; treatments compared with control or as indicated. $^{\dagger}p < 0.05$; $^{\#}p < 0.001$ F (250 μM)-, Dex (250 nM)-, F + Dex (250 μM + 250 nM)-treated cells in the absence or present of fisetin.

in cytotoxicity when compared to single factor exposure, an effect reversed by fisetin. We found that fisetin (60 μM) completely abrogates cytotoxicity of F and Dex co-treatment in both cell lines (Fig. 2). However, fisetin at similar concentration did not entirely inhibit the apoptosis induced by similar treatment. This discrepancy may be due to the fact that in contrast to flow cytometry MTT assay does not distinguish between early apoptosis, late apoptosis and necrosis. Interestingly, Machalinska et al. (2001) found that NaF induced apoptosis in human bone marrow cells. Apoptosis has been also demonstrated by Xiang et al. (2005) to play an important role in the neurotoxicity of excessive fluoride intake. However, Liu et al. (2007) found that 2.5–10.0 μg/ml NaF did not induce apoptosis in mouse fetal long bone cultures.

Dexamethasone can also induce apoptosis as shown by Kao et al. (2010) in iPS cell-derived osteocyte-like cells. Furthermore, it has been demonstrated that glucocorticoids induce apoptosis in primary osteoblast cultures and other cell lines including OCT-1, C3H10T1/2 osteoblastic cells and MLO-Y4 osteocytic cells (Ahuja et al., 2003; Liu et al., 2004). However, glucocorticoids have been shown to protect primary osteoblasts and osteoblastic cell lines (MC3T3-E1, MG63) from apoptosis and this effect may be dependent on cell culture conditions (Zalavras et al., 2003).

Mechanisms of F-induced apoptosis may be multifaceted. For example, Ren et al. (2011) observed that F affects apoptosis through insulin-like growth factor-I receptor (IGF-IR) in mouse osteoblasts. Some authors suggested that both fluoride and dexamethasone induce apoptosis through caspase-dependent pathway (Aden et al., 2008; Chrysis et al., 2005; Gutiérrez-Salinas et al., 2010).

Other mechanisms involved in F- and Dex-induced cell death and apoptosis may be generation of reactive oxygen and nitrogen species (Murphy et al., 2011). Reactive oxygen species are produced under physiological conditions during metabolic reactions. However, excessive ROS production is associated with cellular dysfunction and contributes to the pathogenesis of various human diseases (Analía et al., 2008; Halliwell and Gutteridge, 2006; Inkielewicz-Stepniak and Czarnowski, 2010; Mittal and Flora, 2007; You et al., 2009). Many agents, which are oxidant or stimulators of cellular oxidative metabolism play a major role in various forms of cell death, including apoptosis. Therefore, we have investigated whether co-exposure to F and Dex of osteoblast and hippocampal cells could be associated with generation of ROS. We found that both compounds induced the generation of ROS in MC3T3-E1 and HT22 cells, and the effect was attenuated by fisetin (Fig. 4).

The findings of F-induced ROS generation in osteoblast and hippocampal cells are compatible with our previous studies which showed that F causes lipid and protein peroxidation in the brain of rats exposed to NaF in drinking water (Inkielewicz-Stepniak and Czarnowski, 2010; Krechniak and Inkielewicz, 2005). Furthermore, Zhang et al. (2008) reported that F elevated a level of ROS in the primary rat hippocampal neurons in a dose-dependent manner.

Similarly, the link between glucocorticoid exposure and ROS generation has been reported before. Analía et al. (2008) have found that dexamethasone has a neurotoxic action on hippocampus rats receiving 9 daily subcutaneous injections of dexamethasone (0.5 mg/kg). Moreover, You et al. (2009) have suggested that excess glucocorticoids cause hippocampal damage by regulating genes involved in ROS generation. Our results indicate that co-exposure to F and Dex will result in amplification of ROS generation and increased cytotoxicity. Indeed, ROS can cause cellular damage directly, by attacking proteins, and indirectly, by generating further reactive species and initiating radical chain reactions (Halliwell and Gutteridge, 2006).

It is also known that $H_2O_2$ is mainly responsible for nonenzymatic oxidation of DCFH (LeBel et al., 1992). We found that the F- and Dex-induced apoptosis is attenuated by catalase emphasizing the role of ROS in this process (Fig. 5).

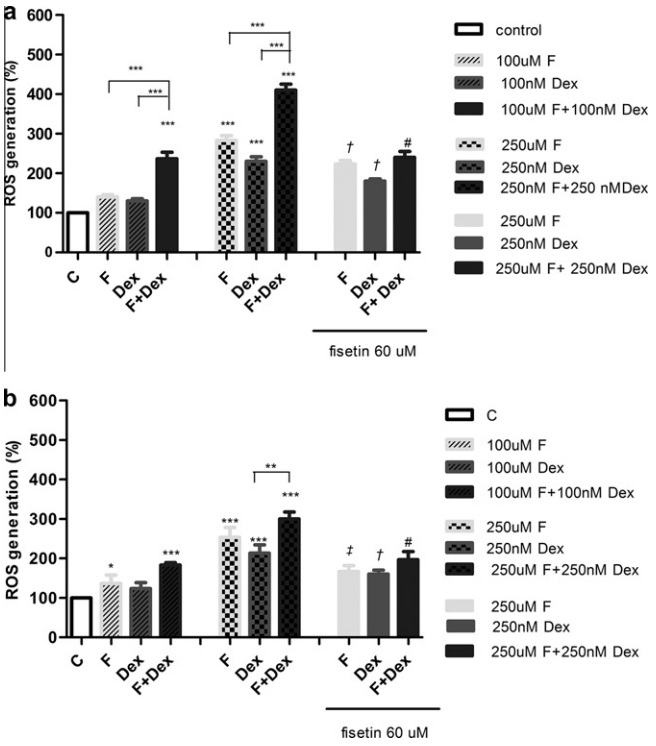


**Fig. 4.** Increased generation of ROS in MC3T3-E1 and HT22 cells following exposure to F, Dex, F + Dex and protective effect of fisetin. Fisetin inhibits F-, Dex-, F + Dex-induced ROS generation in MC3T3-E1 (a) and HT22 (b). Data are as means ± SD of 3–4 separate determinations. $^{**}p < 0.01$; $^{***}p < 0.001$; treatments v/s control or as indicated. $^{\dagger}p < 0.05$; $^{\#}p < 0.001$ F (250 μM)-, Dex (250 nM)-, F + Dex (250 μM + 250 nM)-treated cells in the absence or present of fisetin.

Many studies have concluded that the production of NO has important roles in the regulation of both hippocampal and osteoblast metabolism (Danziger et al., 1997; Steinert et al., 2010). NO is a messenger and effector molecule that mediates diverse biological functions, such as vasodilation, platelet homeostasis, neurotransmission, and immunity; however, excessive production of NO leads to cytotoxic effects (Halliwell and Gutteridge, 2006; Inkielewicz-Stepniak and Czarnowski, 2010; Krechniak and Inkielewicz, 2005; Zhang et al., 2008). Much of NO-mediated pathogenicity depends on the formation of secondary intermediates such as peroxynitrite anion and nitrogen dioxide that are more reactive and toxic than NO *per se* (Radi et al., 2000). The formation of reactive nitrogen species from NO requires the presence of oxidants such as superoxide radicals and hydrogen peroxide ($H_2O_2$) (Halliwell and Gutteridge, 2006; Radi et al., 2000). It has been reported that exposition to F leads to overproduction of NO in tissues (Inkielewicz-Stepniak and Czarnowski, 2010; Zhang et al., 2008). Indeed, Gao et al. (2007) reported that a high dose of F might persistently induce the expressions of iNOS and catalyse synthesis of NO leading to regulation of osteoblast and osteoclast activity and bone turnover. As expected we found that F induced generation of NO in both cell lines. In contrast, Dex did not significantly affect NO levels (Fig. 6).

This is because glucocorticoids are known to inhibit the expression of iNOS (Kleinert et al., 1996; Korhonen et al., 2002; Radomski et al., 1990). Co-incubation of F with Dex resulted in generation of NO. This indicated that under our experimental conditions F-induced generation of NO is iNOS-independent and may be mediated by stimulation of constitutive isoforms of NO synthase. Bergandi et al. (2011) showed using immunoblotting that after a 24 h incubation human osteoblast cells with NaF there was no expression of iNOS or nNOS, while the expression of eNOS was evident. Interestingly, F-induced generation of NO was reduced by fisetin (Fig. 6)

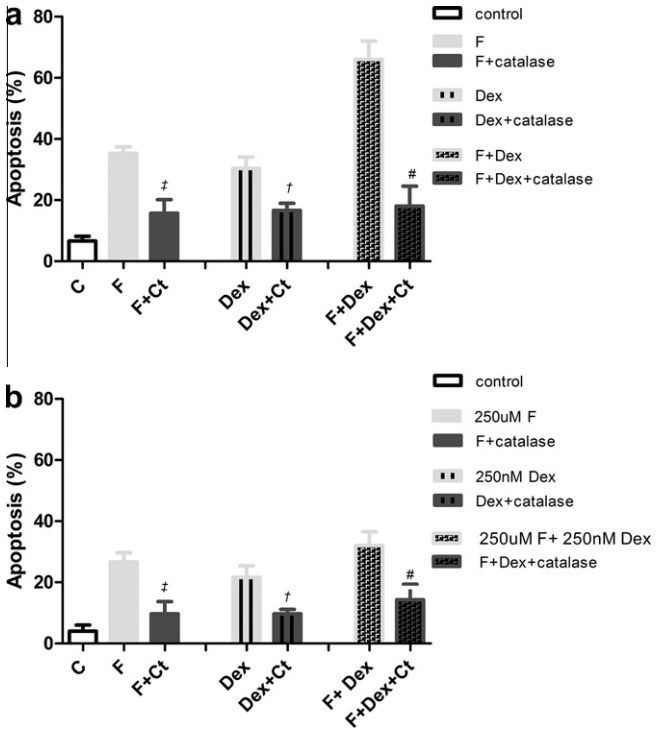


**Fig. 5.** Reversal of F-, Dex- and F and Dex-induced apoptosis by catalase. Effect of F (250 μM), Dex (250 nM), F + Dex (250 μM + 250 nM) on apoptosis in MC3T3-E1 (a) and HT22 (b) with the absence and presence of catalase (400 units/ml). Data are presented as means ± SD of 3–4 separate determinations. $^{\dagger}p < 0.05$; $^{\ddagger}p < 0.01$; $^{\#}p < 0.001$ F-, Dex-, F + Dex-treated cells in the presence or absence of catalase.

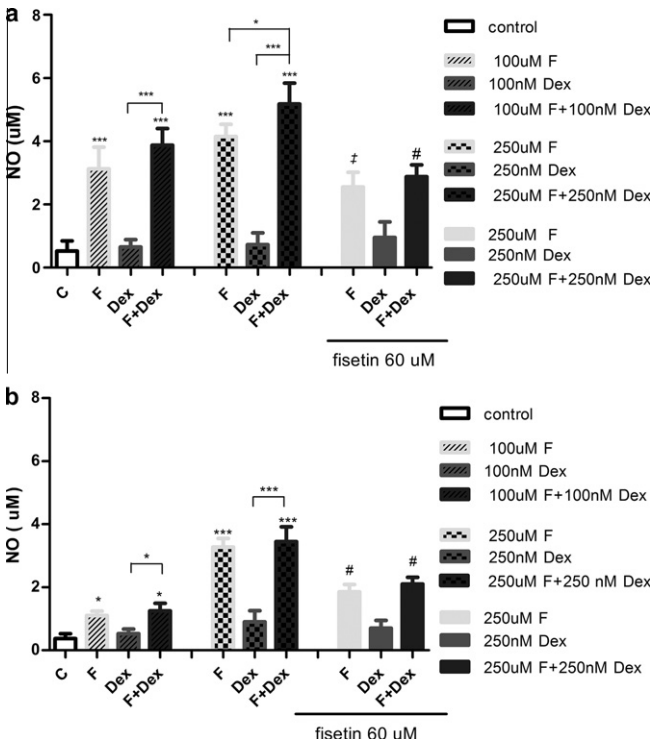


**Fig. 6.** Fluoride-induced generation of NO in MC3T3-E1 and HT22 cells and its inhibition by fisetin. Effects of fisetin of F, Dex, F + Dex-induced NO levels in MC3T3-E1 (a) and HT22 (b). Data are means ± SD of 3–4 separate determinations. $^{**}p < 0.01$; $^{***}p < 0.001$; treatments compared with control or as indicated. $^{\ddagger}p < 0.01$; $^{\#}p < 0.001$ F (250 μM)-, Dex (250 nM)-, F + Dex (250 μM + 250 nM)-treated cells in the absence or present of fisetin.

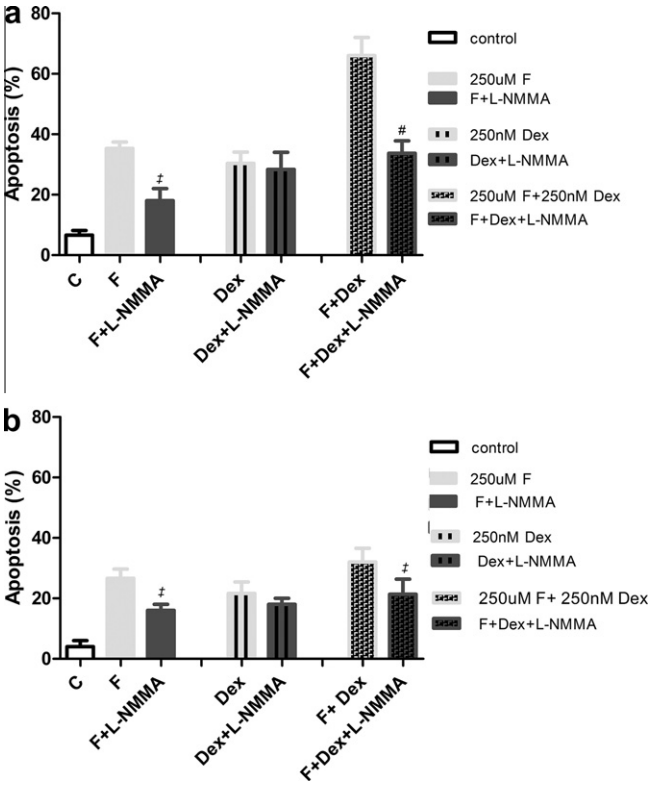


**Fig. 7.** Reversal of F- and F + Dex-induced apoptosis by L-NMMA. Effect of F (250 μM), Dex (250 nM), F + Dex (250 μM + 250 nM) on apoptosis in MC3T3-E1 (a) and HT22 (b) with the absence and presence of L-NMMA (1 mM). Data are means ± SD of 3–4 separate determinations. $^{\ddagger}p < 0.01$; $^{\#}p < 0.001$ F-, Dex-, F + Dex-treated cells in the presence or absence of L-NMMA.

indicating that the flavonoid may act as a NO scavenger or affect NO synthase activity. Indeed, Wang et al. (2006) have demonstrated that inhibitory effects of fisetin on NO production in murine macrophages RAW 264.7 cells were accompanied by a dose-dependent suppression of the gene expression of iNOS. Similar result was found Zheng et al. (2008) in lippolysaccharide-stimulated BV-2 microglia cells and primary microglia cultures. Wallerath et al. (2005) noticed that fisetin did not change eNOS expression or eNOS promoter activity in any substantial way in human EA.hy 926 endothelial cells. To probe directly the significance of F-induced NO generation for cell apoptosis we used L-NMMA, a selective inhibitor of NOS. The treatment with L-NMMA decreased F-but not Dex-induced apoptosis underscoring the role of NO in this process (Fig. 7). We found that pre-incubation with catalase and L-NMMA together; prior to fluoride treatment completely inhibit apoptosis (Fig. 8). These results clearly indicate that NO and hydrogen peroxide cause fluoride-induced apoptosis in both cell lines and discount the involvement of other apoptotic stimuli.

The effects of fisetin, observed during our experiments, may have an important pharmaceutical significance. In the recent years an explosive growth of research on various bioactive flavonoids with therapeutic activities as well as high potency and low toxicity has been observed. Much attention has focused nowadays on the potential uses of flavonoid-based drugs for the prevention and therapy of free radical mediated tissues damage and human diseases. Oxidative stress activates apoptosis, and antioxidants protect against apoptosis *in vitro*; thus, a central role of dietary antioxidants may be to protect against apoptosis (Khan et al., 2008; Sengupta, 2004; Woodman and Chan, 2004). Several studies suggested that fisetin can be used as a nutraceutical or functional food with cytoprotective capacity (Khan et al., 2008; Lian et al., 2008; Woodman and Chan, 2004). Lee et al. (2011) have found that fisetin pretreatment reduced

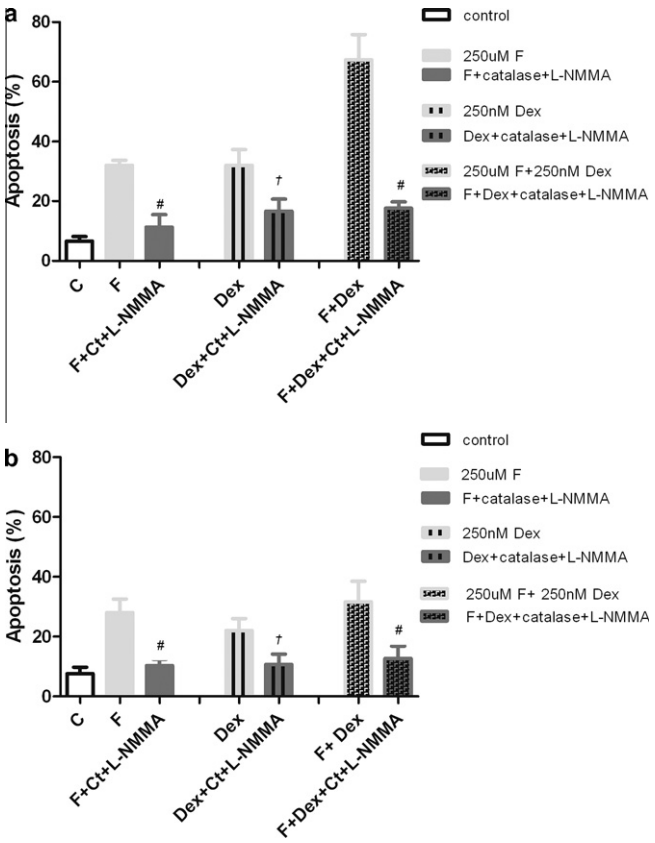


**Fig. 8.** Reversal of F-, and F + Dex-induced apoptosis by catalase + L-NMMA. Effect of F (250 μM), Dex (250 nM), F + Dex (250 μM + 250 nM) on apoptosis in MC3T3-E1 (a) and HT22 (b) with the absence and presence of catalase + L-NMMA (400 units/ml and 1 mM, respectively). Data are presented as means ± SD of 3 separate determinations. $^{\dagger}p < 0.05$; $^{\#}p < 0.001$ F-, Dex-, F + Dex-treated cells in the presence or absence of catalase and L-NMMA.

hydrogen peroxide-induced cell death. Wätjen et al. (2005) have noticed that low concentration (10–25 μM) of fisetin protects cells against damage, whereas high concentrations (50–250 μM) induced apoptosis. However, we have not observed this dependency. Our study demonstrates, for the first time, that fisetin can attenuate fluoride- and dexamethasone-induced oxidative/nitrosative damage in osteoblast and hippocampal cell lines.

## 4. Conclusion

In conclusion, our work shows that co-exposition to fluoride and dexamethasone for 3 days enhanced ROS generation, increased NO level and apoptosis in MC3T3-E1 and HT22 cells and clarifies the role of $H_2O_2$ and NO in cytotoxicity of these xenobiotics. The obtained data illustrated also that the treatment with fisetine reduced these toxic effects. Therefore, our study has given promising results indicating that fisetin can be useful in protection from F- and Dex-mediated bone and brain damage.

## Conflict of Interest

The authors declare that there are no conflicts of interest.

## Acknowledgment

This research was supported by the Grant W-134 from Medical University in Gdansk.

## References

Aden, P., Goverud, I., Liestøl, K., Løberg, E.M., Paulsen, R.E., Maehlen, J., Lømo, J., 2008. Low-potency glucocorticoid hydrocortisone has similar neurotoxic effects as high-potency glucocorticoid dexamethasone on neurons in the immature chicken cerebellum. Brain Research 1236, 39–48.
Ahuja, S.S., Zhao, S., Bellido, T., Plotkin, L.I., Jimenez, F., Bonewald, L.F., 2003. CD40 ligand blocks apoptosis induced by tumor necrosis factor alpha, glucocorticoids, and etoposide in osteoblasts and the osteocyte-like cell line murine long bone osteocyte-Y4. Endocrinology 144, 1761–1769.
Analía, M.F., Ramiro, F., Nicolás, F., Juan, I.R., Roberto, C., Daniel, P.C., 2008. Neuroprotective effect of melatonin on glucocorticoid toxicity in the rat hippocampus. Open Physiological Journal 1, 23–27.
Bergandi, L., Aina, V., Malavasi, G., Morterra, C., Ghigo, D., 2011. The toxic effect of fluoride on MG-63 osteoblast cells is also dependent on the production of nitric oxide. Chemico-Biological Interactions 190, 179–186.
Boulos, P., Ioannidis, G., Adachi, J.D., 2000. Glucocorticoid-induced osteoporosis. Current Rheumatology Reports 2, 53–61.
Chrysis, D., Zaman, F., Chagin, A.S., Takigawa, M., Sävendahl, L., 2005. Dexamethasone induces apoptosis in proliferative chondrocytes through activation of caspases and suppression of the Akt-phosphatidylinositol 3'-kinase signaling pathway. Endocrinology 146, 1391–1397.
Chua, C.C., Chua, B.H., Chen, Z., Landy, C., Hamdy, R.C., 2003. Dexamethasone induces caspase activation in murine osteoblastic MC3T3-E1 cells. Biochimica et Biophysica Acta 1642, 79–85.
Cohen, D., Adachi, J.D., 2004. The treatment of glucocorticoid-induced osteoporosis. Journal of Steroid Biochemistry and Molecular Biology 88, 337–349.
Conradie, M.M., de Wet, H., Kotze, D.D., Burrin, J.M., Hough, F.S., Hulley, P.A., 2007. Vanadate prevents glucocorticoid-induced apoptosis of osteoblasts in vitro and osteocytes in vivo. Journal of Endocrinology 195, 229–240.
Danziger, R.S., Zuckerbraun, B.S., Pensler, J.M., 1997. Role of nitric oxide in the regulation of osteoblast metabolism. Plastic and Reconstructive Surgery 100, 670–673.
Gao, Y., Sun, D., Zhou, L., Yu, J., Li, Y., Wang, Y., 2007. Effect of subchronic fluorosis on iNOS expression in rat bone tissue. Fluoride 40, 297–298.
Gourlay, M., Franceschini, N., Sheyn, Y., 2007. Prevention and treatment strategies for glucocorticoid-induced osteoporotic fractures. Clinical Rheumatology 26, 144–153.
Gutiérrez-Salinas, J., Morales-González, J.A., Madrigal-Santillán, E., Esquivel-Soto, J., Esquivel-Chirino, C., González-Rubio, M.G., Suástegui-Domínguez, S., Valadez-Vega, C., 2010. Exposure to sodium fluoride produces signs of apoptosis in rat leukocytes. International Journal of Molecular Sciences 11, 3610–3622.
Halliwell, B., Gutteridge, J.M.C., 2006. Free Radicals in Biology and Medicine, fourth ed. Oxford University Press, New York.
Inkielewicz-Stepniak, I., Czarnowski, W., 2010. Oxidative stress parameters in rats exposed to fluoride and caffeine. Food and Chemical Toxicology 48, 1607–1611.
Kao, C.L., Tai, L.K., Chiou, S.H., Chen, Y.J., Lee, K.H., Chou, S.J., Chang, Y.L., Chang, C.M., Chen, S.J., Ku, H.H., Li, H.Y., 2010. Resveratrol promotes osteogenic differentiation and protects against dexamethasone damage in murine induced pluripotent stem cells. Stem Cells Deviation 19, 247–258.
Khan, N., Afaq, F., Syed, D.N., Mukhtar, H., 2008. Fisetin, a novel dietary flavonoid, causes apoptosis and cell cycle arrest in human prostate cancer LNCaP cells. Carcinogenesis 29, 1049–1056.
Kimira, M., Arai, Y., Shimoi, K., Watanabe, S., 1998. Japanese intake of flavonoids and isoflavonoids from foods. Journal of Epidemiology 8, 168–175.
Kleinert, H., Euchenhofer, C., Ihrig-Biedert, I., Forstermann, U., 1996. Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-κB. Molecular Pharmacology 49, 15–21.
Korhonen, R., Lahti, A., Hämäläinen, M., Kankaanranta, H., Moilanen, E., 2002. Dexamethasone inhibits inducible nitric-oxide synthase expression and nitric oxide production by destabilizing mRNA in lipopolysaccharide-treated macrophages. Molecular Pharmacology 62, 698–704.
Krechniak, J., Inkielewicz, I., 2005. Correlations between fluoride concentration and free radical parameters in soft tissues of rats. Fluoride 38, 293–296.
Krishnamachari, K.A., 1986. Skeletal fluorosis in humans: a review of recent progress in the understanding of the disease. Program of Food Nutrition Science 10, 279–314.
LeBel, C.P., Ischiropoulos, H., Bondy, S.C., 1992. Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chemical Research in Toxicology 2, 227–231.
Lee, S.E., Jeong, S.I., Yang, H., Park, C.S., Jin, Y.H., Park, Y.S., 2011. Fisetin induces Nrf2-mediated HO-1 expression through PKC-δ and p38 in human umbilical vein endothelial cells. Journal of Cellular Biochemistry. doi:10.1002/jcb.23158.
Lian, T.W., Wang, L., Lo, Y.H., Huang, I.J., Wu, M.J., 2008. Fisetin, morin and myricetin attenuate CD36 expression and oxLDL uptake in U937-derived macrophages. Biochimica et Biophysica Acta 1781, 601–609.
Liu, Y., Porta, A., Peng, X., Gengaro, K., Cunningham, E.B., Li, H., Dominguez, L.A., Bellido, T., Christakos, S., 2004. Prevention of glucocorticoid-induced apoptosis in osteocytes and osteoblasts by calbindin-D28k. Journal of Bone and Mineral Research 19, 479–490.
Liu, K., Ma, L., Yao, H., Zhang, Y., Li, L., Wang, G., 2007. Fluoride-mediated apoptosis and disordering of cell cycle distributions during in vitro organ culture of mouse fetal long bones. Fluoride 40, 19–23.
Lu, Y., Sun, Z.R., Wu, L.N., Wang, X., Lu, W., Liu, S.S., 2000. Effect of high-fluoride water on intelligence in children. Fluoride 33, 74–78.

Machalinska, A., Machoy-Mokrzynska, A., Marlicz, W., Stecewicz, I., Machalinskia, B., 2001. NaF-induced apoptosis in human bone marrow and cord blood CD34 positive cells. Fluoride 34, 258–263.
McEwen, B.S., de Leon, M.J., Lupien, S.J., Meaney, M.J., 1999. Corticosteroids, the aging brain and cognition. Trends in Endocrinology and Metabolism 10, 92–96.
Mittal, M., Flora, S.J., 2007. Vitamin E supplementation protects oxidative stress during arsenic and fluoride antagonism in male mice. Drug and Chemical Toxicology 30, 263–281.
Murphy, M.P., Holmgren, A., Larsson, N.G., Halliwell, B., Chang, C.J., Kalyanaraman, B., Rhee, S.G., Thornalley, P.J., Partridge, L., Gems, D., Nyström, T., Belousov, V., Schumacker, P.T., Winterbourn, C.C., 2011. Unraveling the biological roles of reactive oxygen species. Cell Metabolism 13, 361–366.
Ozaki, T., Habara, K., Matsui, K., Kaibori, M., Kwon, A.H., Ito, S., Nishizawa, M., Okumura, T., 2010. Dexamethasone inhibits the induction of iNOS gene expression through destabilization of its mRNA in proinflammatory cytokine-stimulated hepatocytes. Shock 33, 64–69.
Park, H.S., Lim, J.H., Kim, H.J., Choi, H.J., Lee, I.S., 2007. Antioxidant flavone glycosides from the leaves of Sasa borealis. Archives Pharmacy Research 30, 161–166.
Radi, R., Denicola, A., Alvarez, B., Ferrer-Sueta, G., Rubbo, H., 2000. Nitric oxide biology and pathobiology. In: Ignarro, L. (Ed.), Nitric Oxide. Academic Press, Inc., San Diego, pp. 57–82.
Radomski, M.W., Palmer, R.M.J., Moncada, S., 1990. Glucocorticoids inhibit the expression of an inducible, but not constitutive nitric oxide synthase in vascular endothelial cells. Proceedings of the National Academy of Sciences of the United States of America 87, 10043–10047.
Ren, G., Ferreri, M., Wang, Z., Su, Y., Han, B., Su, J., 2011. Sodium fluoride affects proliferation and apoptosis through insulin-like growth factor I receptor in primary cultured mouse osteoblasts. Biological Trace Element Research. doi:10.1007/s12011-011-9059-0.
Rich, C., Ensinck, J., 1961. Effect of sodium fluoride on calcium metabolism of human beings. Nature 191, 184–185.
Roma, L.P., Bosqueiro, J.R., Cunha, D.A., Carneiro, E.M., Gurgul-Convey, E., Lenzen, S., Boschero, A.C., Souza, K.L., 2009. Protection of insulin-producing cells against toxicity of dexamethasone by catalase overexpression. Free Radical Biology and Medicine 47, 1386–1393.
Sapolsky, R.M., 1990. Glucocorticoids, hippocampal damage and the glutamatergic synapse. Progress in Brain Research 86, 13–23.
Sengupta, P.K., 2004. Investigations on the binding and antioxidant properties of the plant flavonoid fisetin in model biomembranes. FEBS Letters 570, 77–81.
Steinert, J.R., Chernova, T., Forsythe, I.D., 2010. Nitric oxide signaling in brain function, dysfunction, and dementia. Neuroscientist 16, 435–452.
Tome, M.E., Briehl, M.M., 2001. Thymocytes selected for resistance to hydrogen peroxide showaltered antioxidant enzyme profiles and resistance to dexamethasone induced apoptosis. Cell Death and Differentiation 8, 953–961.
Vyvey, M., 2010. Steroids as pain relief adjuvants. Canadian Family Physician 56, 1295–1297.
Wallerath, T., Li, H., Gödtel-Ambrust, U., Schwarz, P.M., Förstermann, U., 2005. A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. Nitric Oxide 12, 97–104.
Wang, L., Tu, Y.C., Lian, T.W., Hung, J.T., Yen, J.H., Wu, M.J., 2006. Distinctive antioxidant and antiinflammatory effects of flavonols. Journal of Agricultural and Food Chemistry 54, 9798–9804.
Wang, Z., Yang, X., Yang, S., Ren, G., Ferreri, M., Su, Y., Chen, L., Han, B., 2011. Sodium fluoride suppress proliferation and induce apoptosis through decreased insulin-like growth factor-I expression and oxidative stress in primary cultured mouse osteoblasts. Archives of Toxicology. doi:10.1007/s00204-011-0697-y.
Wätjen, W., Michels, G., Steffan, B., Niering, P., Chovolou, Y., Kampkötter, A., Tran-Thi, Q.H., Proksch, P., Kahl, R., 2005. Low concentrations of flavonoids are protective in rat H4IIE cells whereas high concentrations cause DNA damage and apoptosis. Journal of Nutrition 135, 525–531.
WHO, 2002. Fluorides. World Health Organization, Geneva.
WHO, 2003. Drugs and Therapeutic Committee – A Practical Guide. World Health Organization, Geneva.
Woodman, O.L., Chan, E.Ch., 2004. Vascular and anti-oxidant actions of flavonols and flavones. Clinical and Experimental Pharmacology and Physiology 31, 786–790.
Xiang, Q.Y., Wang, C.S., Liang, Y.X., 2005. Toxicity of fluoride on nervous system. Chinese Journal of Endemiology 24, 114–116.
Yan, X., Feng, C., Chen, Q., Li, W., Wang, H., Lv, L., Smith, G.W., Wang, J., 2009. Effects of sodium fluoride treatment in vitro on cell proliferation, apoptosis and caspase-3 and caspase-9 mRNA expression by neonatal rat osteoblasts. Archives of Toxicology 83, 451–458.
You, J.M., Yun, S.J., Nam, K.N., Kang, C., Won, R., Lee, E.H., 2009. Mechanism of glucocorticoid-induced oxidative stress in rat hippocampal slice cultures. Canadian Journal of Physiology and Pharmacology 87, 440–447.
Zalavras, C., Shah, S., Birnbaum, M.J., Frenkel, B., 2003. Role of apoptosis in glucocorticoid-induced osteoporosis and osteonecrosis. Critical Reviews in Eukaryotic Gene Expression 13, 221–235.
Zhang, M., Wang, A., Xia, T., He, P., 2008. Effects of fluoride on DNA damage, S-phase cell-cycle arrest and the expression of NF-B in primary cultured rat hippocampal neurons. Toxicology Letters 179, 1–5.
Zheng, L.T., Ock, J., Kwon, B.M., Suk, K., 2008. Suppressive effects of flavonoid fisetin on lipopolysaccharide-induced microglial activation and neurotoxicity. International Immunopharmacology 8, 484–494.