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# **The Effect of Epigallocatechin Gallate (EGCG) and Metal Ions Corroded from Dental Casting Alloys on Cell Cycle Progression and Apoptosis in Cells from Oral Tissues**

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## **1. Introduction**

### **1.1 The application of dental metallic materials**

Metallic materials are the basis of dental materials science, which is an important part of dentistry. As a result of their good mechanical properties and some biological properties, metals are widely used in oral rehabilitation of dentition and mandibular defects, orthodontic and dental implants, as well as in the equipment for dental operations. Commonly used metals include nickel chromium, cobalt chromium, titanium, and silver palladium alloy; of these, nickel-chromium and cobalt-chromium alloys are in widespread clinical use in China because of their stable biological properties and moderate prices.

The oral environment is a very complex electrolyte environment with a large number of microorganisms, in which the metal prosthesis corrodes and releases metal ions. Microbial corrosion is a major contributor to this process. The products of bacterial metabolism (including organic and inorganic acids) can directly affect the pH on the surface or interface of a metal prosthesis, and thus affect the electrochemical reactivity, eventually promoting corrosion. Metal ions are increased in the saliva and oral soft tissue (e.g. gingiva & tongue smear) of patients who wear alloy prostheses. The metal ions released from dental casting alloys can potentially reduce the metabolism of cells, inhibit cell proliferation and have other toxic effects in vitro. Ions released from commonly used Ni-Cr alloys may induce adverse reactions, such as gingival inflammation and discoloration, oral cell mutation or cancer. Cobalt ions from Co-Cr alloy have been reported to stimulate allergic reaction. Another ion that may be released, molybdenum, though is not a main component of Co-Cr alloy, can potentially cause mutagenesis, inactive some important enzymes, such as alkaline phosphatase, and inhibit chromium absorption via leukocytes.

### **1.2 The biological effects of dental casting alloys**

Microbial corrosion refers to electrochemical reactions aroused or catalyzed by microorganisms, rather than the metal specifically corroded by microorganisms. Microbial

corrosion requires appropriate conditions for microbial reproduction, and is often the result of multi-microbial symbiosis and interaction. In the oral environment, dental casting alloys are sensitive to micro-organisms and tarnish, e.g. nickel, chromium, copper, aluminum, iron, palladium and zinc. It is reported that *Actinomyces viscosus* has a significant influence on the electrochemical corrosion of Ni-Cr alloy and Gold alloy; the existence of *streptococcus mutans* increases the corrosion of Ni-Cr alloy. On the other hand, oral bacteria can produce lactic acid and acetic acid. When fluoride ions, hydrogen peroxide and lactic acid exist simultaneously in the mouth, the corrosion of titanium would greatly increase. The biggest corrosion threat to iron is glucose, followed by acetic acid (Park et al., 2007). As mentioned above, the oral bacteria play an important role in material deterioration. Oral microorganisms should be considered when discussing biocompatibility of prostheses.

In conjunction with the corrosion process of dental casting alloys, many metal ions are absorbed within the digestive system. Per unit ion concentration,  $K^+$ ,  $Cd^{2+}$ ,  $V^{2+}$ ,  $Ag^+$ ,  $Hg^{2+}$ ,  $Sb^{3+}$ ,  $Be^{2+}$ , and  $In^{3+}$  have higher toxicity, while  $Sn^{4+}$ ,  $Zr^{4+}$ ,  $Nb^{5+}$ , and  $Mo^{6+}$  have lower toxicity.  $Cr^{6+}$  and  $Be^{2+}$  have the highest toxicity towards the cell membrane integrity and protein synthesis of human gingival fibroblasts;  $Ni^{2+}$  is moderately toxic to these cells, and  $Cr^{3+}$  and  $Mo^{6+}$  show the lowest toxicity.  $Ni^{2+}$ ,  $Co^{2+}$ ,  $Ti^{4+}$ , and  $V^{3+}$  could affect DNA synthesis, ALP (alkaline phosphatase) activity and calcification processes of osteoblast-like cells in vitro. Bone marrow cells can be significantly damaged when exposed to  $Cr^{6+}$  for 48h;  $Co^{2+}$ ,  $Mo^{6+}$ , and  $Ni^{2+}$  also showed moderate toxicity;  $V^{5+}$  showed significant toxicity after 4 weeks.

The commonly used Ni-Cr alloy (which also contains Be) could cause gingival fibroblast morphology, viability and proliferation changes, increase the production of PCNA (Proliferating cell nuclear antigen) and Bcl-2 (B-cell lymphoma gene 2) proteins, and boost the production of IL-6, IL-8 and other cytokines involved in inflammation. These effects show positive correlations with concentration of Co, Cr and Cu ions. After wearing Ni-Cr alloy (with Be), animal experiments demonstrated apoptosis lymphocytes and DNA damage of buccal epithelial cells, which deteriorated further with increased wearing time; the gold alloy in contrast, produced no side effects. (Su et al., 2006a, 2006b, 2006c, 2008a, 2008b; Yu et al., 2007)

## 2. The tea application

Tea, which originated in China, and has spread over the world, has become a popular worldwide drink. Nowadays, over 34 countries produce tea, and there are more than 4 billion tea consumers in over 100 countries.

Tea is not only a refreshing drink, but also a healthy drink for the prevention of radiation sickness, cardiovascular diseases and cancer, with certain pharmacological effects. The main active ingredients in tea are tea polyphenols, which are strong antioxidants. These polyphenolic compounds mainly consist of catechins. They have a health benefits, including lowering blood cholesterol and blood pressure, and anti-cancer, anti-bacterial and anti-viral effects.

### 2.1 Biological effects of catechin

The green tea catechins make up approximately 60–80 wt.% of tea polyphenols. (-)-epigallocatechin gallate (EGCG) is the most abundant of the four major catechins which also include (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epicatechin (EC).

EGCG is also the most active component of green tea leaves. It has been shown to have biological effects, including antimutagenicity, antitumorigenesis, free radical scavenging, etc. Epidemiologic studies demonstrated that a low rate of tumorigenesis may be associated with the habit of drinking tea. Experiments in animal models and cells also suggested that green tea extract may contribute to the inhibition of the generation and development of tumors. In addition to the above-mentioned effect, EGCG, as reported, plays a critical role in inducing apoptosis of malignant cells, regulating key elements of signal pathway and inhibiting telomerase activity.

## 2.2 Biological effects of EGCG

EGCG is a potent antioxidant component that has demonstrated great antioxidant protection in experimental studies. It is also involved in protecting against free-radical DNA damage, and interferes with the binding of cancer-causing agents to cellular DNA. Besides, EGCG plays a role in inhibition of lipid peroxidation and inducement of apoptosis of malignant cells by regulating various signal pathways. For instance, EGCG may induce apoptosis of cells through binding to the antiapoptotic proteins Bcl-2 and Bcl-xL;

EGCG can negatively regulate protein serine/threonine phosphatase-2A (PP-2A) to positively regulate p53-dependent apoptosis. In addition: EGCG induces apoptosis of JB6 cells, which is associated with hyperphosphorylation of p53 and up-regulation of the proapoptotic gene, Bak (Qin et al., 2008); the interruption of the VEGF (vascular endothelial growth factor) signaling pathway by EGCG results in caspase activation and subsequent malignant cell death; EGCG induces stress signals by damaging mitochondria and ROS-mediated JNK activation in MIA PaCa-2 pancreatic carcinoma cells; EGCG-mediated caspase activation induces proteolytic cleavage of the NF-kappa B/p65 subunit, leading to the loss of transactivation domains, and driving the cells towards apoptosis; EGCG show its cytotoxicity to PC-3 cells by up-regulating the 67LR and the mitochondria-mediated apoptosis pathway (Zhang et al., 2008); and finally, EGCG treatment resulted in dose-dependent inhibition of TNF- $\alpha$ -induced production of MMP-1 and MMP-3 at the protein and mRNA levels in RA synovial fibroblasts (Yun et al., 2008).

The mechanism of the biological effects of EGCG may differ for different cell categories. Treatment of PC-3 cells with EGCG resulted in time and concentration-dependent activation of the extracellular signal-regulated kinase (ERK1/2) pathway. In contrast, EGCG treatment did not induce ERK1/2 activity in RWPE-1 cells (Albrecht et al., 2008). In HLE cells (hepatoma cell line), EGCG induced apoptosis, but not cell-cycle arrest, and appears to have down-regulated Bcl-2 $\alpha$  and Bcl-xl by inactivation of NF-kappaB. Oral administration of EGCG showed similar effects in HLE xenograft tumors; in normal human primary epidermal keratinocytes (NHEK), one of the key mediators of EGCG action is p57/KIP2, a cyclin-dependent kinase (CDK) inhibitor. EGCG potently induces p57 in NHEK, but not in epithelial cancer cells. It is c-Jun N-terminal kinase (JNK) signaling that mediates EGCG-induced apoptosis, and exogenous expression of p57 suppresses EGCG-induced apoptosis via inhibition of JNK.

Research has indicated that the 67-kDa laminin receptor (67LR) mediates epigallocatechin gallate (EGCG)-induced cell growth inhibition and reduction of myosin regulatory light chain (MRLC) phosphorylation at Thr-18/Ser-19, which is important for cytokinesis through

67LR. EGCG at a physiological concentration can activate myosin phosphatase by reducing myosin phosphatase 1 (MYPT1) phosphorylation and that may be involved in EGCG-induced cell growth inhibition (Umeda et al., 2008). Matrix metalloproteinase (MMP-9) expression is linked with myeloid cell differentiation, as well as inflammation and angiogenesis processes related to cancer progression. EGCG inhibited MMP-9 secretion in a time- and dose-dependent manner. The gene and protein expression of MMP-9 and of the mRNA stabilizing factor HuR were also inhibited. In an invasion assay, EGCG repressed the invasion of lung carcinoma, and it down-regulated the expression of MMP-9. NF-kappa B localized in the nucleus of the 95-D cells was diminished in a dose-dependent manner in EGCG-treated cells. Thus, the inhibition of tumor invasion by EGCG was shown to be attributed to decreases in the expression of MMP-9 and NF-kappa B, which may result from decrease of intracellular oxidants (Annabi et al. 2007).

EGCG induced dose- and time-dependent apoptotic cell death accompanied by loss of mitochondrial transmembrane potential, release of cytochrome c into the cytosol, and cleavage of pro-caspase-9 to its active form; EGCG also enhanced production of intracellular reactive oxygen species (ROS) (Noda et al., 2007).

### 3. Tea and metal ions

#### 3.1 The effect of tea polyphenols on the corrosion behavior of dental casting alloys

Ni-Cr alloy has become prevalent in oral prosthetic applications; although many instances of side effects caused by fixed nichrome dentures have been reported. Gingival inflammation and the appearance of marginal gingival blackening are both ascribed to release of metal ions from the denture. Thus, we wondered if the habit of drinking tea affected the corrosion behavior of dental casting alloys.

As reported, the recommended daily intake of tea is 6-16 g per person, and it differs with age. The major component of green tea, tea polyphenol, accounts for 20-30 wt.% of the dry weight of green leaves. The concentration of weak tea (4-6 g/L green tea) is equivalent to 1.25 g/L tea polyphenol solution, while strong tea (17-25 g/L green tea) is about 5 g/L. An average consumption of green tea (8-13g/L green tea) equates to 2.5g/L tea polyphenol solution.

Experiments showed that the effect of tea polyphenol on Ni-Cr alloy was the most obvious; it enhanced the corrosion rate of Ni-Cr alloy at low concentrations. Tea polyphenol at a concentration of 1.25 g/L had the lowest corrosion resistance compare to the other two groups (the alloy treated with 2.5g/L and 5g/L tea polyphenol ).

With an increase in the concentration of tea polyphenol, the corrosion resistance of Ni-Cr alloy improved. A surface view of Ni-Cr alloy with a scanning electron microscope demonstrated that the surface of the alloy treated with 1.25 g/L tea polyphenol was the roughest, compared with the other samples. It was a very interesting result. Although the exact principle is unclear, the EGCG could formulate an insoluble chelate with Ni and/or Cr, thus precluding further corrosion.

Co-Cr alloy possesses good hardness and high strength, and more importantly does not contain adverse elements as Be and Ni. A great deal of scientific research has demonstrated that the corrosion of the alloy with Cr (>16%) is reduced because of the existence of a



passive film on the surface. Treatment with a high concentration of tea polyphenol could further reduce the corrosion tendency of the Co-Cr alloy. Compared with in artificial saliva, the Co-Cr alloy in tea polyphenol has a lower corrosion tendency and corrosion rate.

Drinking tea at the recommended daily intake is helpful to enhance the corrosion resistance of Co-Cr alloy. Thus, it is advised that people with Co-Cr alloy prostheses increase their intake of tea polyphenol. This will give the prosthesis a long service life.

As titanium has good corrosion resistance, it is more and more widely used in clinical stomatology. In tea polyphenol solutions, with increasing concentration of polyphenols, the corrosion of titanium accelerates as its corrosion resistance decreases. Therefore, while lower concentrations of tea help to increase the corrosion resistance of titanium, if the concentration is much higher than the recommended amount for daily drinking the stability of titanium will be lower, as described above, with potentially harmful effects. So patients wearing titanium dentures should not drink concentrated tea solution.

We have studied both artificial saliva, and three different concentrations of polyphenol solutions. Among the three alloys studied, the corrosion rate of the titanium is slowest (i.e. its corrosion resistance is best); the corrosion rate of the nickel-chromium alloy is fastest (so its corrosion resistance is worst, and showed the most serious corrosion in the experiments) and the corrosion resistance of cobalt-chromium alloy is between that of titanium and nickel-chromium alloy.

### 3.2 Complexation reactions of tea polyphenols and metal ions

Polyphenols that are abundant in green tea contain multiple hydroxyl groups. As a result, green tea has a strong acid-base buffering capacity, and the polyphenols can complex with central ions such as  $\text{Ni}^{2+}$ ,  $\text{Bi}^{3+}$ ,  $\text{Cr}^{6+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Al}^{3+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mo}^{6+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Mn}^{2+}$ , and form chelate rings. A number of studies show that flavonoids are strong chelating agents with  $\text{Fe}^{3+}$ . Catechins (flavonols) contain epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC). All of them can form complexes with  $\text{Fe}^{3+}$ . A "B ring" catechol group is required for combination of polyphenols and  $\text{Fe}^{3+}$ ; when the B ring of 3,4 o-hydroxy changes into a 3,4,5-trihydroxy gallic acyl group, the efficiency of the combination of polyphenol and  $\text{Fe}^{3+}$  is low. Although gallic acid (another phenolic acid) can form complexes with  $\text{Fe}^{3+}$ , the binding capacity of the polyphenols is weak because of the galloyl functional group. The functional groups and chemical characterization of polyphenols and metal ions have been studied for a long time. Chemical analysis and X-ray diffraction showed that catechin and  $\text{Al}^{3+}$  form a non-crystalline precipitate complex with a ratio of 1:1. Nuclear magnetic resonance spectroscopy shows the chemical shifts of catechins change upon complex formation; infrared absorption spectra show that the absorption bands of some functional groups of epicatechin disappeared

Some experiments show that catechins folded into a cavity-like structure around  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$ , capturing  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$  through the ring  $\pi$ -electron clouds of the aromatic compounds and forming complexes with 1:1 ratios. However, many studies suggest tea polyphenols and metal ions can coordinate in a wide variety of ways. The coordination modes of tea polyphenols and metal ions change with changing pH. At different pH values, catechin and  $\text{Fe}^{3+}$  form complexes in different proportions: when  $\text{pH} < 3$ ,  $\text{Fe}^{3+}$  and catechin combined at a molar ratio of 1:1; when  $3 < \text{pH} < 7$ , the molar ratio was 2:1; and when  $\text{pH} > 7$ ,

the molar ratio was 4:1. The stability constants of these complexes are in the range  $10^5$ – $10^{17}$  (Sungur & Uzar, 2007). Electrospray mass spectrometry shows that catechins and iron ions form three different compounds ( $L^1Fe$ ,  $L^2Fe$  and  $L^3Fe$  with one to three ligands) at different pH values (Elhabiri et al., 2007). Different kinds of polyphenols also have different coordination modes with metal ions. Studies show that EGC and EC combined with  $Mn^{2+}$  through the o-hydroxy of the B ring, while EGCG mainly combines with  $Mn^{2+}$  through the D ring (gallic ring), and then through the B ring (gallate catechin ring), as the coordination bond formed at the D-loop was stronger than that at the B ring. The same tea polyphenols and metal ions can form different complexes in different coordination modes. To form  $[Al(egcgH-2)]^+$ , two hydroxyl groups on the D-ring of epigallocatechin gallate become deprotonated, while to form  $[Al(egcgH-3)]^0$ , first one hydroxyl group on the B-ring and two on the D-ring are deprotonated, then  $Al^{3+}$  combines with two oxygen atoms on the D-ring and one on the B-ring to form a polymeric structure.

In addition, oxidation-reduction reaction can also take place between tea polyphenols and metal ions. The reduction potential of tea polyphenols is high, so it is easy for them to auto-oxidize under certain conditions. High valence metal ions can oxidize the polyphenols to their quinone or other derivatives, with the metal ions being reduced to their lower valent states. Catechins, the main components of tea polyphenols, have been shown experimentally to reduce  $Cu^{2+}$  and  $Fe^{3+}$  into  $Cu^+$  and  $Fe^{2+}$ . Because the  $Cu^{2+}/Cu^+$  redox potential is low, the  $Cu^{2+}/Cu^+$  reaction is easier than the  $Fe^{3+}/Fe^{2+}$  reaction. When excess  $Fe^{3+}$  was added into gallic acid (GA),  $Fe^{3+}$  rapidly combined with the o-hydroxyl on the B-ring of gallic acid, and formed complexes at the ratio of 1:1, as indicated by the formation of a dark blue solution. Subsequently, other complexes can formed electron transfer reactions -  $Fe^{3+}$  was reduced to  $Fe^{2+}$ , and GA was oxidized to semiquinone, which then rapidly combined with the remaining  $Fe^{3+}$  to form benzoquinone.

## 4. Effects of interaction between EGCG and metal ions on cells

### 4.1 Effects of interaction between EGCG and metal ions on cell proliferation rate

We found that EGCG has a strong inhibitory effect on the growth of tongue squamous carcinoma cells; with significant inhibition beginning at 100  $\mu M$ . In contrast, for gingival fibroblasts, a small inhibition of cell growth was noted at over 150  $\mu M$  EGCG (Fig. 1). Some studies have shown that  $Ni^{2+}$  has carcinogenic effects, and some have shown that its toxicity and carcinogenic effects are related to its absorption, transport, distribution and retention in cells. Our studies show that when the concentration of EGCG is less than 150  $\mu M$ , the growth of gingival fibroblasts remains almost unaffected (cell survival rate is more than 95%); when EGCG concentration ranges from 150  $\mu M$  to 300  $\mu M$ , a concentration- and time-dependent inhibition of growth of gingival fibroblasts is seen. These results indicated that EGCG may have little promotion on the growth of gingival fibroblasts at low concentrations, and strong inhibition at high concentrations (Fig. 1). Interestingly, we also found that the interactions between EGCG and  $Ni^{2+}/Co^{2+}$  enhanced the inhibition effect of EGCG on cell growth (Fig. 2, Fig. 4), as well as cell morphological changes.  $Cr^{3+}/Mo^{6+}$  have little effect on the growth of tongue squamous carcinoma cells and gingival fibroblast cells, and the interaction of EGCG and  $Cr^{3+}/Mo^{6+}$  produces no significant inhibition effects on tongue squamous carcinoma cells and gingival fibroblast cells (Fig. 3, Fig. 5).

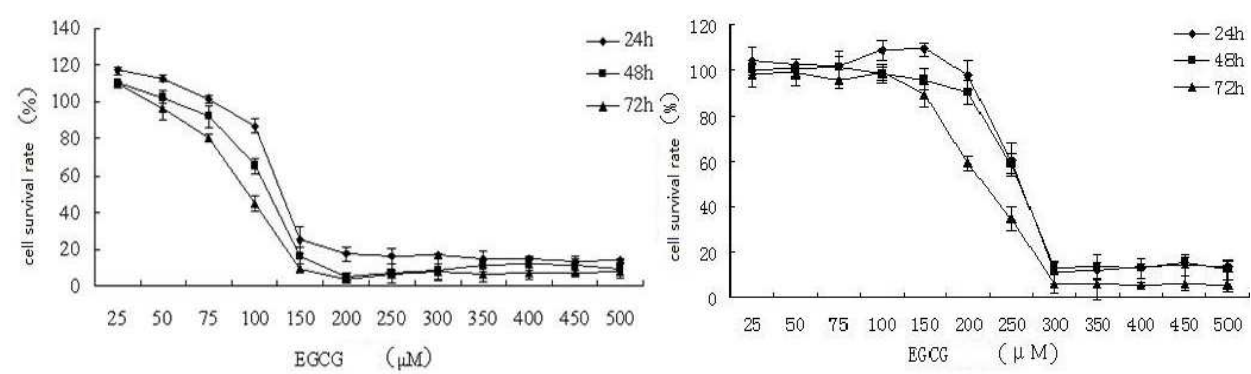


Fig. 1. Cell survival rates in tongue squamous cancer Cal-27 cells (left) and human gingival fibroblast cells (right) upon treatment with EGCG. The cell density was detected at 24, 48 and 72 h after cell seeding. Cell survival rate was calculated as the number of cells present at these time points compared with the initial cell seeding density. Data points represent the mean  $\pm$  SD for each group

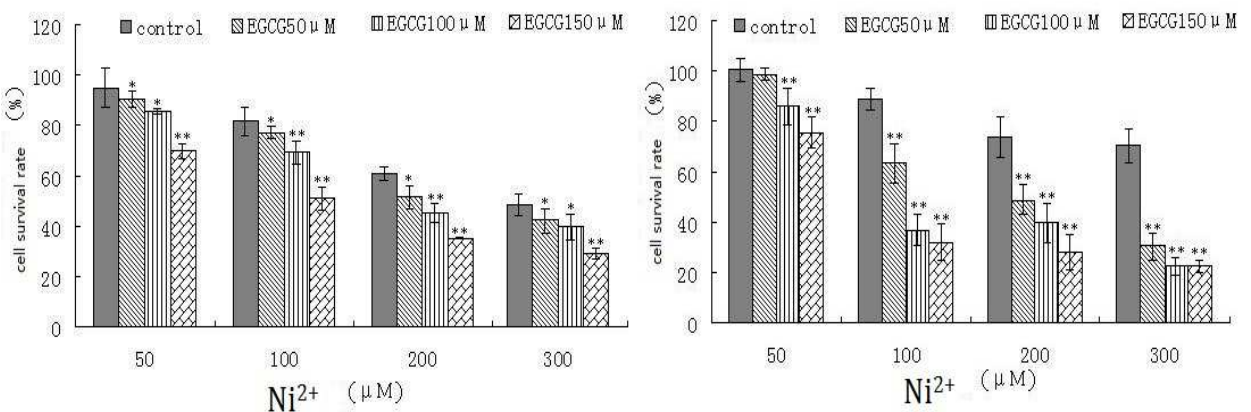


Fig. 2. Cell survival rates in tongue squamous cancer Cal-27 cells (left) and human gingival fibroblast cells (right) upon treatment with combinations of EGCG and Ni<sup>2+</sup> for 48 hours compared with the control group (treatment with Ni<sup>2+</sup> only). Data points represent the mean  $\pm$  SD. n=6 (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ )

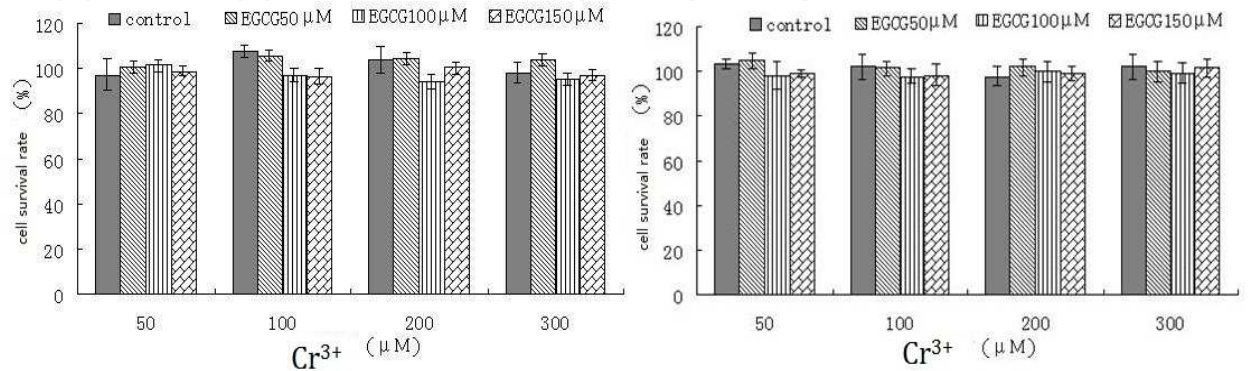


Fig. 3. Cell survival rates in tongue squamous cancer Cal-27 cells (left) and human gingival fibroblast cells (right) upon treatment with combinations of EGCG and Cr<sup>3+</sup> for 48 hours compared with the control group (treatment with Cr<sup>3+</sup> only). Data points represent the mean  $\pm$  SD. n=6 (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ )



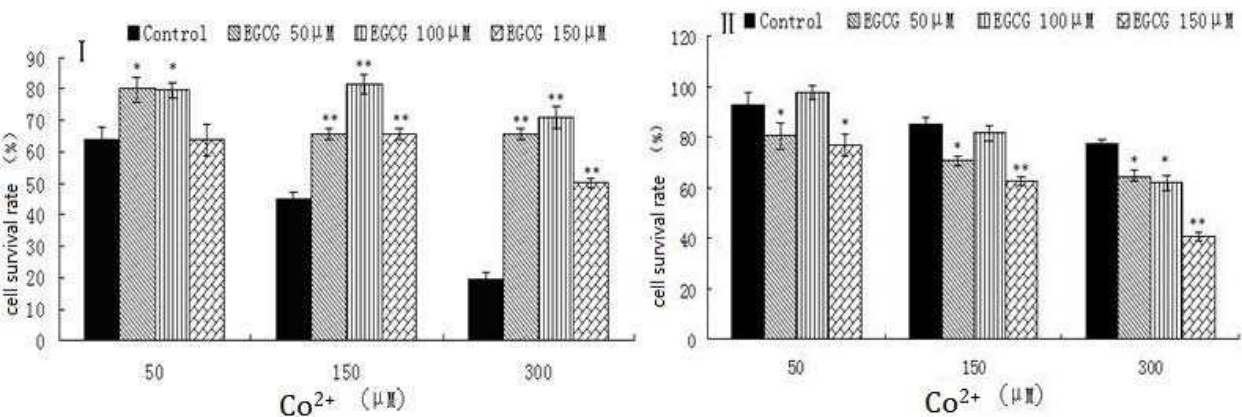


Fig. 4. Cell survival rates in tongue squamous cancer Cal-27 cells (left) and human gingival fibroblast cells (right) upon treatment with combinations of EGCG and Co<sup>2+</sup> for 48 hours. The control group was treated with Co<sup>2+</sup> only. Data points represent the mean ± SD. n=6 (\*, p < 0.05; \*\*, p < 0.01)

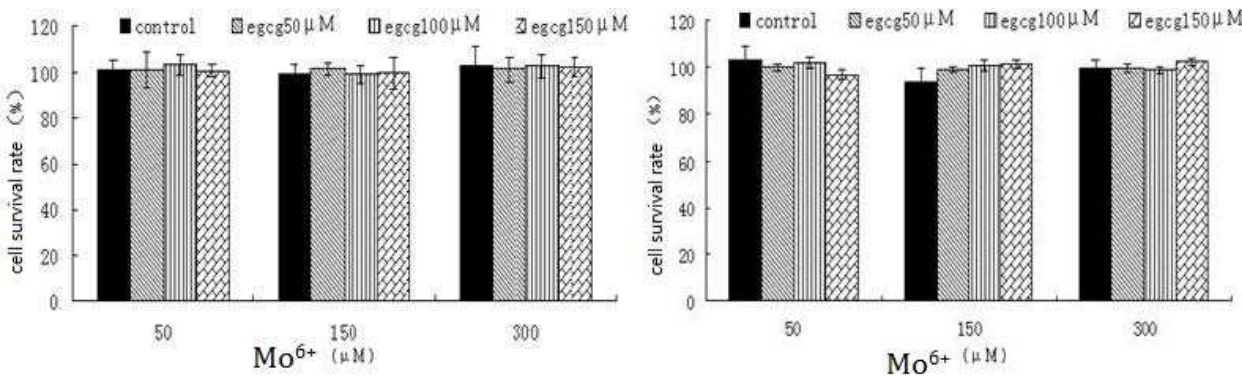


Fig. 5. Cell survival rates in tongue squamous cancer Cal-27 cells (left) and human gingival fibroblast cells (right) upon treatment with combinations of EGCG and Mo<sup>6+</sup> for 48 hours compared with the control group (treatment with Mo<sup>6+</sup> only). Data points represent the mean ± SD. n=6 (\*, p < 0.05; \*\*, p < 0.01)

4.2 The influence of the combination of metal ions and EGCG on cellular DNA

DNA is a biological macromolecule, which occupies a central and critical role in the cell as its genetic information. A variety of factors can cause DNA damage in cells, such as ionizing radiation, peroxides, thiols and certain metal ions. It has been reported that Ni<sup>2+</sup> can decrease DNA synthesis, change the structure of DNA, reduce protein synthesis and inhibit DNA replication and transcription. It can also cause decrease of succinate dehydrogenase activity and total cellular protein. In addition, Ni<sup>2+</sup> complexes with nitrogen compounds may damage DNA in breast cancer cells (Lü et al., 2009). However, Cr<sup>3+</sup> is difficult to get into cells, and hence has no significant effect on the human gingival fibroblast cells (Elshahawy et al., 2009).

Single cell gel electrophoresis (SCGE), also called comet assay could detect the DNA damage on single cell level. The parameter of Olive Tail Moment reflects both intensity and extent of DNA damage. Ni<sup>2+</sup> in combination with EGCG significantly increased the damage to human gingival fibroblast cells and tongue squamous cancer cells. The damage to tongue squamous

cancer cells could also be increased by the combination of  $\text{Cr}^{3+}$  and EGCG. Furthermore, the damage to human gingival fibroblast cells and tongue squamous cancer cells caused by  $\text{Cr}^{3+}$  in combination with EGCG was significantly less than that produced by 100  $\mu\text{M}$  EGCG alone (Figs. 6–7).

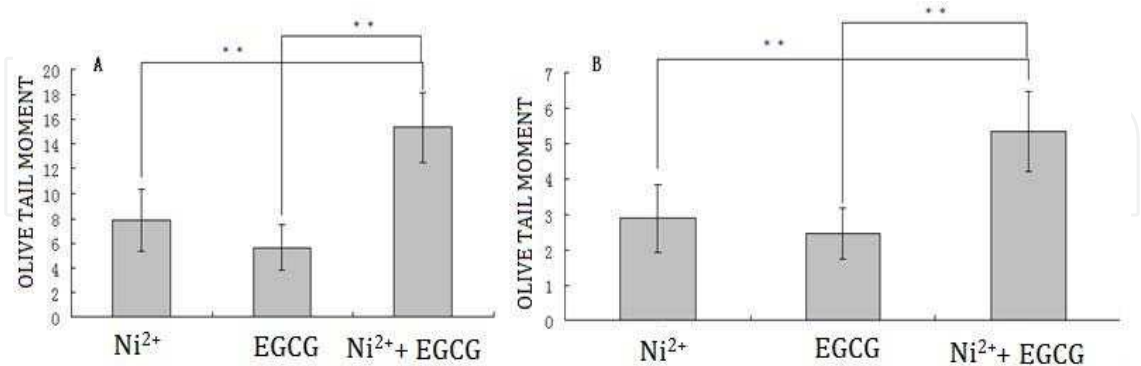


Fig. 6. Comet Assay: DNA damage in tongue squamous cancer Cal-27 cells (left) and human gingival fibroblast cells (right) after treatments with EGCG,  $\text{Ni}^{2+}$  and EGCG combined with  $\text{Ni}^{2+}$ . Cells were treated with 100  $\mu\text{M}$  EGCG and/or 200  $\mu\text{M}$   $\text{Ni}^{2+}$  for 48 hours

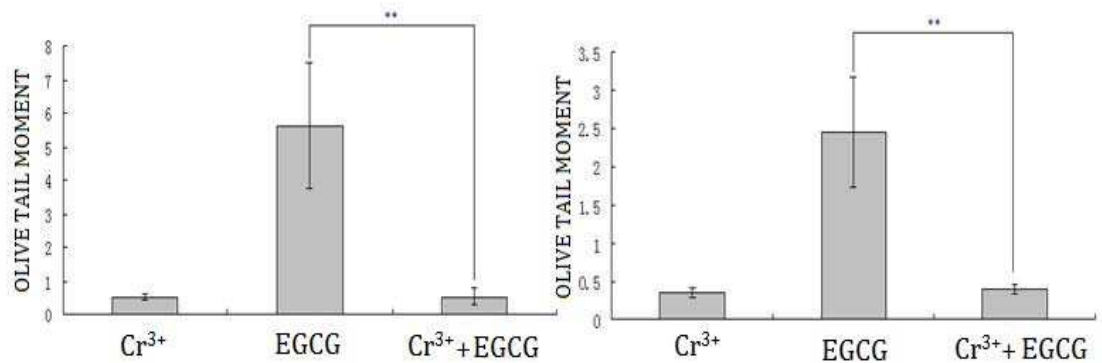


Fig. 7. Comet Assay: DNA damage in tongue squamous cancer Cal-27 cells (left) and human gingival fibroblast cells (right) after treatments with EGCG,  $\text{Cr}^{3+}$  and EGCG combined with  $\text{Cr}^{3+}$ . Cells were treated with 100  $\mu\text{M}$  EGCG and/or 200  $\mu\text{M}$   $\text{Cr}^{3+}$  for 48 hours

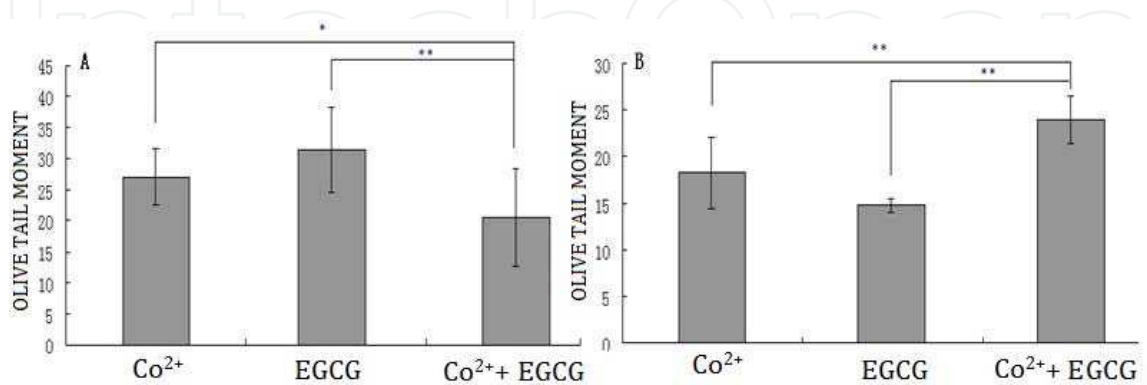


Fig. 8. Comet Assay: DNA damage in tongue squamous cancer Cal-27 cells (left) and human gingival fibroblast cells (right) after treatments with EGCG,  $\text{Co}^{2+}$  and EGCG combined with  $\text{Co}^{2+}$ . Cells were treated with 150  $\mu\text{M}$  EGCG and/or 300  $\mu\text{M}$   $\text{Co}^{2+}$  for 48 hours

The combination of  $\text{Co}^{2+}$  and EGCG produced less DNA damage than either individual component on tongue squamous cancer cells, but increased the damage to human gingival fibroblast cell DNA.  $\text{Mo}^{6+}$  in combination with EGCG produced significantly less damage than EGCG alone to both human gingival fibroblast cells and tongue squamous cancer cells (Figs. 8–9).

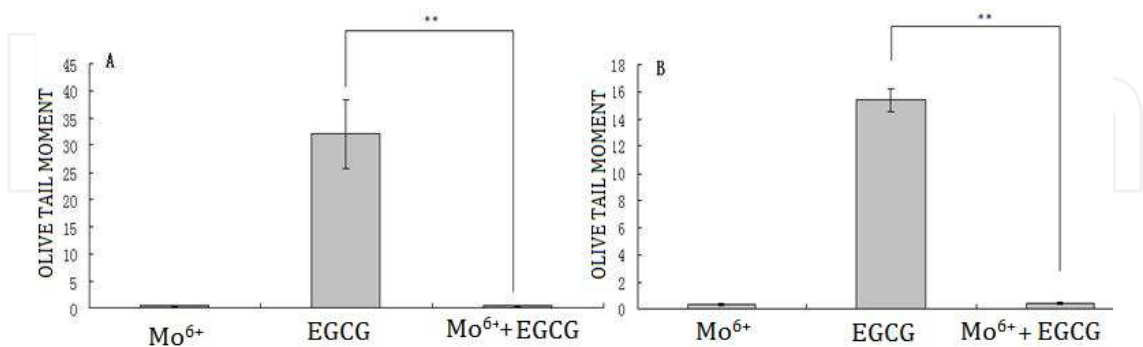


Fig. 9. Comet Assay: DNA damage in tongue squamous cancer Cal-27 cells (left) and human gingival fibroblast cells (right) after treatments with EGCG,  $\text{Mo}^{6+}$  and EGCG combined with  $\text{Mo}^{6+}$ . Cells were treated with 150  $\mu\text{M}$  EGCG and/or 300  $\mu\text{M}$   $\text{Mo}^{6+}$  for 48 hours

4.3 The influence of interaction of metal ions and EGCG on cell cycle

Flow cytometry (FCM) is an efficient experimental method for cell cycle detection and estimation of apoptotic cells. Hence, we use FCM to measure the apoptosis. The result

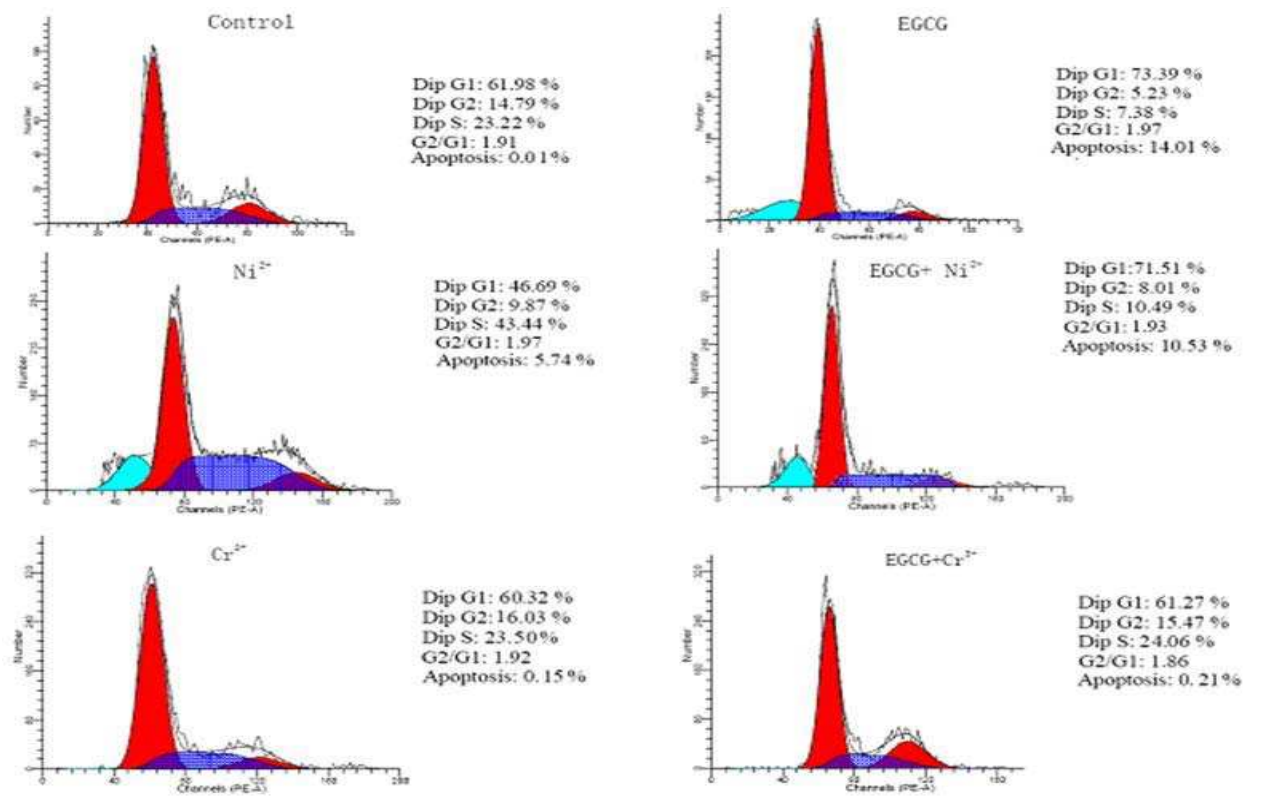


Fig. 10. Cell cycles for tongue squamous cancer Cal-27 cells during treatment with EGCG,  $\text{Ni}^{2+}$ ,  $\text{Cr}^{3+}$ , EGCG combined with  $\text{Ni}^{2+}$  and EGCG combined with  $\text{Cr}^{3+}$

showed that: EGCG induces G0/G1 cell cycle arrest and partial apoptosis in the tongue squamous cancer cells.  $\text{Ni}^{2+}$  arrests the cell cycle at S in the human gingival fibroblast cells and tongue squamous cancer cells. After exposure to the combination of EGCG and  $\text{Ni}^{2+}$ , the cell cycle is arrested at G0/G1 and apoptosis is increased. Compared with the  $\text{Ni}^{2+}$  induced effect, the effect of the combination of  $\text{Ni}^{2+}$  and EGCG is enhancement of the apoptosis of both human gingival fibroblast cells and tongue squamous cancer cells. This indicates that the cytotoxicity of  $\text{Ni}^{2+}$  enhanced after co-treatment with  $\text{Ni}^{2+}$  and EGCG. In contrast,  $\text{Cr}^{3+}$  has no obvious influence on the cell cycle of the human gingival fibroblast cells and tongue squamous cancer cells. The effect on the cell cycle of human gingival fibroblast cells and tongue squamous cancer cells induced by EGCG could be reduced by combination of  $\text{Cr}^{3+}$  and EGCG. (Figs. 10-11)

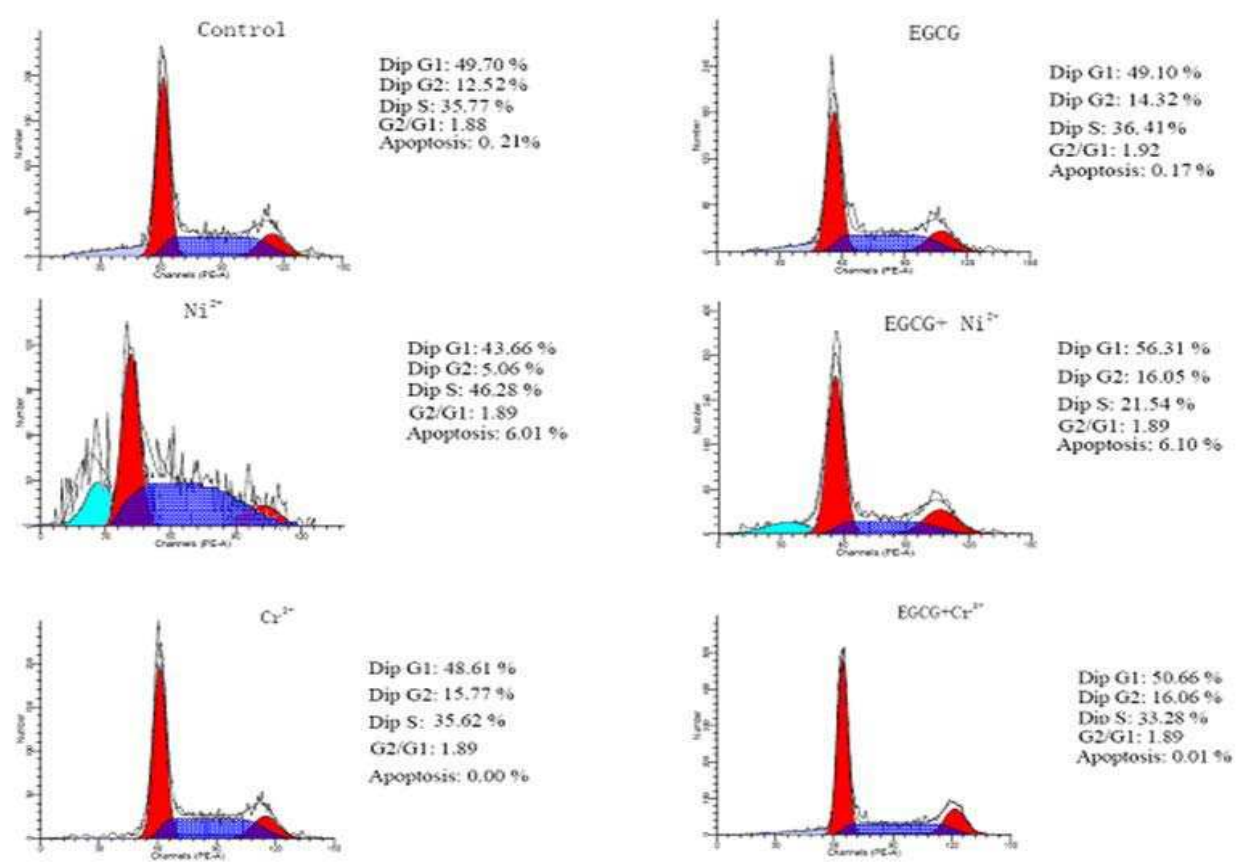


Fig. 11. Cell cycles for human gingival fibroblast cells during treatment with EGCG,  $\text{Ni}^{2+}$ ,  $\text{Cr}^{3+}$ , EGCG combined with  $\text{Ni}^{2+}$  and EGCG combined with  $\text{Cr}^{3+}$

Both EGCG and  $\text{Co}^{2+}$  inhibited the cell cycle of tongue squamous cancer cells, block the DNA synthesis and arrest the cell cycle at G0/G1. However, the combination of EGCG and  $\text{Co}^{2+}$  significantly reduced the inhibition effect caused by EGCG or  $\text{Co}^{2+}$  individually, and increased the percentage of cells in the G2/M phase. There was no significant change in the cell cycle between the group of Cal-27 cells treated with  $\text{Mo}^{6+}$  and the control group, and the cell cycle arrest of Cal-27 cells induced by EGCG could be reduced combination with  $\text{Mo}^{6+}$  to the extent that no significant difference was seen compared with the control group. (Figs. 12-13)



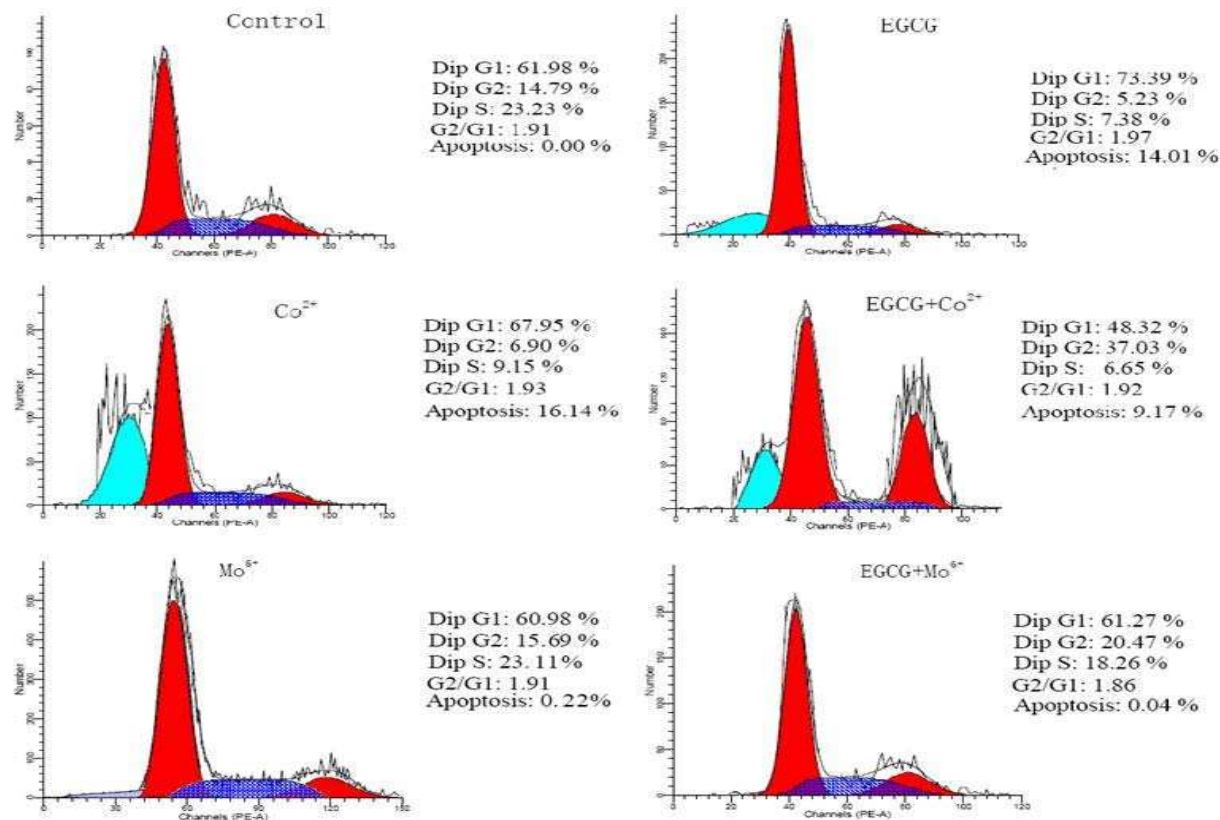


Fig. 12. Cell cycles for tongue squamous cancer Cal-27 cells during treatment with EGCG, Co<sup>2+</sup>, Mo<sup>6+</sup>, EGCG combined with Co<sup>2+</sup> and EGCG combined with Mo<sup>6+</sup>

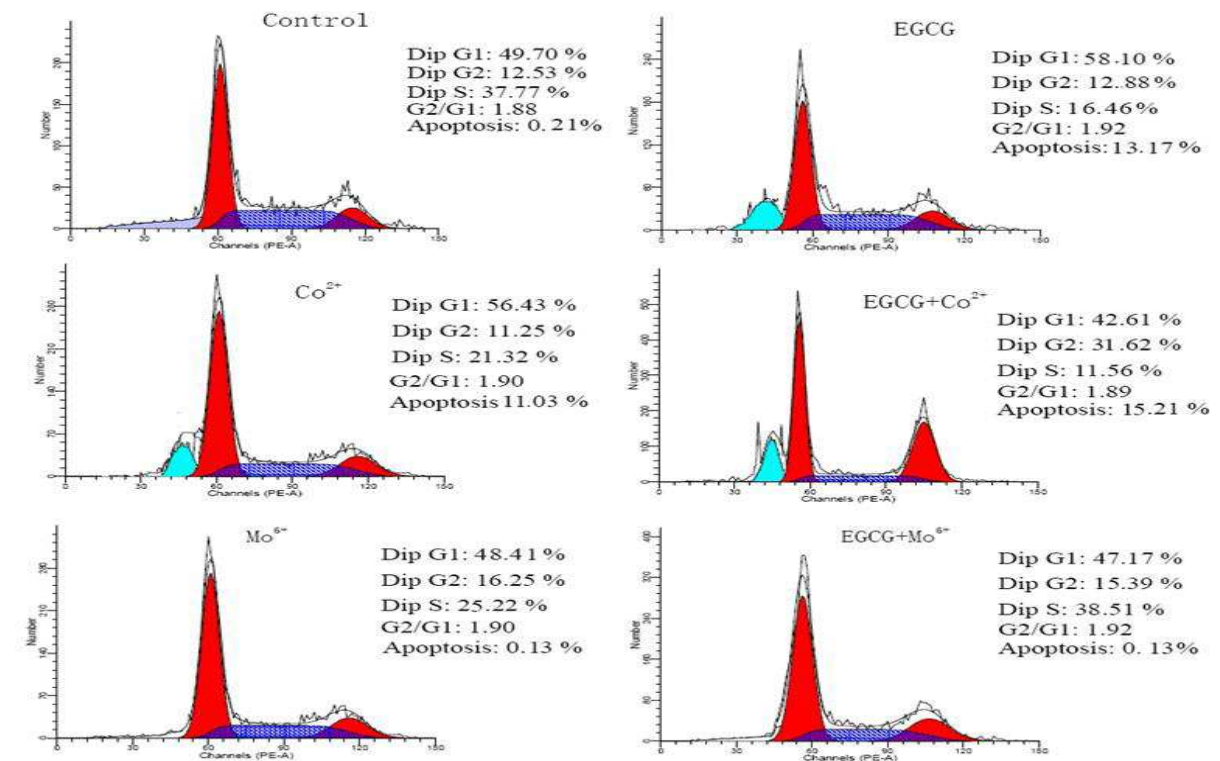


Fig. 13. Cell cycles for human gingival fibroblast cells (right) during treatment with EGCG, Co<sup>2+</sup>, Mo<sup>6+</sup>, EGCG combined with Co<sup>2+</sup> and EGCG combined with Mo<sup>6+</sup>

## 5. Conclusion

Our results show that  $\text{Ni}^{2+}$  in combination with EGCG significantly increases damage to normal gingival fibroblasts and tongue squamous cancer cells, whereas the combination of EGCG and  $\text{Co}^{2+}$  increased damage only to gingival fibroblasts. In contrast, EGCG together with either  $\text{Cr}^{3+}$  or  $\text{Mo}^{6+}$  resulted in significantly less damage than produced by EGCG alone. Moreover, the cell cycle is arrested at G0/G1 and apoptosis after exposure to EGCG+ $\text{Ni}^{2+}$  is increased relative to that occurring after treatment with EGCG only. Combining EGCG+ $\text{Co}^{2+}$  significantly reduced the growth inhibitory effect caused by EGCG or  $\text{Co}^{2+}$  individually, and increased the percentage of cells in the G2/M phase. In contrast, no significant synergistic effect was seen with EGCG+ $\text{Cr}^{3+}$ / $\text{Mo}^{6+}$ .

These results indicate that the toxicity of EGCG may be i) enhanced in the presence of  $\text{Ni}^{2+}$ , ii) diminished when given in combination with  $\text{Cr}^{3+}$  or  $\text{Mo}^{6+}$  or iii) partially inhibited by  $\text{Co}^{2+}$  (although this latter effect is seen only in normal fibroblasts).

Our studies have demonstrated interactions between EGCG and metal ions that affect cell cycle progression and apoptosis. However, more research is required to reveal the binding mode (s) of these substances and the potential mechanisms by which they affect normal and cancerous cells.

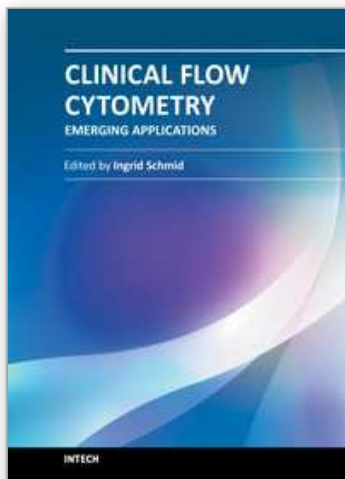
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