Research Article

Procyanidin effects on oesophageal adenocarcinoma cells strongly depend on flavan-3-ol degree of polymerization

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Epidemiological studies have shown that the risk of developing oesophageal adenocarcinoma (OA) is inversely correlated to consumption of fruits and vegetables. Flavan-3-ols are the most abundant subclass of flavonoids in these types of foods. Three apple-derived procyanidin fractions with different average degrees of polymerization (aDP) were characterized and the effects of these fractions and of pure flavan-3-ol monomers ((−)-epicatechin and (+)-catechin) and dimers (B1, B2) on two OA cell lines were investigated. Flavan-3-ol monomers and dimers had no effect on the two cell lines, while apple-derived flavan-3-ol oligomers and polymers induced a time-dependent reduction of cell viability. The reduction in the cell viability was due to the induction of caspase-mediated apoptosis and an arrest of the cell cycle in G0/G1. The magnitude of the reduction in cell viability and induction of apoptosis after exposure to flavan-3-ol oligomeric/polymeric fractions positively correlated with their aDP. These results indicate that only flavan-3-ol oligomers and polymers, but not monomers and dimers, have an effect on the proliferation of OA cells *in vitro*. As tested flavan-3-ol concentrations are achievable through diet, this study suggests that apple-derived PA may possess chemotherapeutic effects against OA.

Keywords: Apoptosis / Cancer / Cell cycle / Flavan-3-ols / Proanthocyanidins

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1 Introduction

The incidence of oesophageal adenocarcinoma (OA) has increased dramatically in Western countries in recent decades [1]. This type of cancer has a poor prognosis, with a mean survival time of less than 1 year [2]. Epidemiological studies have shown that the risk of developing OA is inversely correlated with fruits and vegetables consumption [3–5], and recent work suggests that a high fruit consumption in particular is associated with a reduction in relative risk of as much as 50% [6]. These protective effects do not appear to be directly attributable to conventional micronutrients [7]; much attention has therefore been focussed on the putative antitumourigenic effects of biologically active phytochemicals, the most abundant of which are the flavonoids [8]. Flavan-3-ols are the biggest single flavonoid contributor to dietary intake and are present at high concentration in many plant foods, like apple, cocoa, grape and berries [9].

Flavan-3-ols exist in monomeric form, also known as catechins, and in polymeric form, called proanthocyanidins (PA). The polymeric dimensions of the PA are described by the degree of polymerization (DP), this being the number of flavan-3-ol units present in the polymer. Foods and extracts derived from flavan-3-ol containing plants usually contain a variety of molecules with different polymer dimension and the average degree of polymerization (aDP) is used to describe these fractions. Flavan-3-ols may also be present as gallic acid esters.

Food extracts rich in flavan-3-ols have been shown to reduce cell proliferation and induce apoptosis in various cell lines derived from different types of cancer [10–15]. Also, recent studies have suggested that the presence of gal-

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Abbreviations: aDP, average degree of polymerization; **DP,** degree of polymerization; **JNK,** c-jun N-terminal kinase; **OA,** oesophageal adenocarcinoma; **PA,** proanthocyanidin

loylation increased the magnitude of the flavan-3-ol induced effects on cells [16 – 18]. The oesophagus is the primary site of exposure to dietary flavonoids, however the possible effects of flavan-3-ols against OA have never been assessed. In the present study, we have investigated the effects of nongalloylated flavan-3-ols on two OA cell lines. Cell exposure to flavan-3-ol monomers and dimers and to apple-derived flavan-3-ol fractions with different aDP showed that oligomers and polymers, but not monomers and dimers, reduced cell viability, induced the arrest of the cell cycle in G0/G1 and apoptosis.

2 Materials and methods

2.1 Flavan-3-ol fraction

Purified flavan-3-ol monomers ((–)-epicatechin and (+)-catechin) and dimers (B1, B2) were purchased from Extrasynthese (Genay, France). The flavan-3-ol fractions with aDP 2.3 and 9.2 were obtained from two different apple varieties (Marie Menard and Jeanne Renard, respectively). Sugar and nonphenolic compounds were removed from crude apple extracts using preparative HPLC [19]. The flavan-3-ol fraction with aDP 3.9 was purified from a cider apple (*Malus domestica*, variety Antoniette), as described by Gossé *et al*. [14]. After removing sugar and nonphenolic compounds from the crude apple extract flavan-3-ols were separated from the other phenolic compounds on a fractogel column [20]. Flavan-3-ol unit composition and aDP were characterized by thiolysis coupled with RP-HPLC which is the gold-standard method for measuring these parameters [21]. The three apple-derived fractions consisted of 78 – 95% flavan-3-ols, with (–)-epicatechin the main flavan-3-ol unit (95 – 98% of total flavan-3-ol units). (+)-Catechin accounted for the remaining 2 – 5%. As gallocatechins and catechin gallates were not detected, all the flavan-3-ol oligomers/polymers were procyanidins.

2.2 Normal phase HPLC analysis of apple-derived flavan-3-ol fractions

Flavan-3-ols were separated according to DP by normal phase HPLC using a 5 µm Luna silica column (250×4.6 mm^2) (Pheomenex, Torrance, CA) and a mobile phase consisting of a 65 min gradient as follows: 0 – 30 min, 14.0 – 28.4% B; 30 – 45 min, 28.4 – 39.6% B; 4550 min, 39.6 – 86.0% B; 50 – 55 min, 86.0% B isocratic; 55 – 65 min, 14.0% B; with a constant 4% of C maintained throughout the gradient, where A was dichloromethane, B was methanol and C was acetic acid/water (1:1 v/v). Flavan-3-ols were detected by fluorescence emission at 316 nm, following excitation at 276 nm. Peak areas were integrated following the flat baseline integration method [22] and flavan-3-ol abundance was estimated using published relative response factors [9]; this assumes that the flavan-3-ols in apples give similar responses to those from cocoa, which is likely considering that both sources contain only procyanidins.

2.3 Cell culture

OE-33 and OE-19 human OA cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). The OE-33 cell line was established from a poorly differentiated OA classified as pathological stage IIA (UICC), while the OE-19 cell line was established from a moderately differentiated adenocarcinoma of the gastric cardia/oesophageal gastric junction classified as stage III (UICC). Cells were cultured in monolayers at 37°C in humidified air with 5% CO_2, in RPMI 1640 medium (Gibco, Paisley, UK), supplemented with 10% v/v heat-inactivated foetal bovine serum (Sigma, Poole, UK), penicillin (100 U/mL), streptomycin (100 µg/mL) and glutamine (2 mmol/L) (Sigma). Cells were passaged following treatment with 0.05% trypsin and 0.5 mmol/L EDTA solution (Sigma).

2.4 Cell viability assay

The effects of flavan-3-ols on cell viability were investigated by detecting the accumulation of Neutral Red (Sigma) in adherent monolayers of OA cells following incubation with the compounds. Cells were seeded in 96-well plates, incubated for 48 h to allow cell adhesion, before treatment with pure flavan-3-ol monomers or dimers, or with apple-derived flavan-3-ol fractions. Cell treatments were performed at a final flavan-3-ol concentration of 30 or 60 mg/L for 24 or 48 h. Stocks of flavan-3-ol fractions were dissolved in DMSO and the final DMSO concentration in the medium did not exceed 0.1% v/v. The untreated control consisted of cells grown in culture medium containing 0.1% v/v DMSO. Following exposure to flavan-3-ols, cells were incubated with Neutral Red (0.4% w/v) for 2 h at 37°C, fixed in a 0.5% v/v formaldehyde and 0.1% w/v calcium chloride solution, washed twice with PBS and incubated overnight at 4°C in a 50% v/v ethanol and 1% v/v acetic acid solution. The OD at 550 nm was determined. OE-33 cells were seeded at 10 000 or 6000 cells *per* well for the 24 or 48 h treatments, respectively, similarly OE-19 cells at 11 000 or 8000 cells *per* well. Initial cell concentrations were chosen based on standard growth curve results.

2.5 Cell cycle and apoptosis detection by flow cytometry

OE-33 and OE-19 cells were seeded in 24-well plates, at 80 000 and 60 000 cells *per* well, respectively, and incubated for 48 h. Cells were treated with flavan-3-ol fractions at 60 mg/L flavan-3-ol concentration for 24 or 48 h. To assess the induction of apoptosis, the caspase marker FITC-VAD-

FMK (Promega, WI, USA) was added at 5 μmol/L final concentration to the wells and cells were incubated for 20 min at 37°C. This compound is a fluorescent analogue of a pan-caspase inhibitor that binds to activated caspases, allowing the detection of apoptotic cells at an early stage. Adherent cells were harvested and pooled together with any detached cells. After two washes with PBS, cells were resuspended in 70% v/v cold ethanol and incubated at 4°C for 1 h before staining with propidium iodide (PI), following the instructions of the Coulter DNA-Prep Reagents Kit (Beckman Coulter, UK). Samples were incubated for 30 min at room temperature in a darkened area before analysis using a Coulter EPICS Altra flow cytometer equipped with a water-cooled 488 nm Coherent 90 laser. A minimum of 25 000 events were collected *per* sample. Data acquisition and analysis were performed with Expo32 software (Beckman Coulter).

2.6 Statistical analysis

The statistical significances of differences between means were determined by ANOVA, performed using Minitab release 14 (Minitab, State College, PA).

3 Results

3.1 Characterization of flavan-3-ol oligomers and polymers in apple fractions by normal phase HPLC

Chromatograms obtained from HPLC analyses of the three fractions (Figs. 1A, C and E) and the derived estimations of relative abundance of flavan-3-ols with different degrees of polymerization in each fraction are shown (Figs. 1B, D and F). The flavan-3-ol compositions of three apple fractions were characterized using normal phase HPLC analysis, which allows the separation of flavan-3-ols according to their degree of polymerization. Flavan-3-ols with DP up to seven were separated; flavan-3-ol polymers with DP higher than 10 were eluted as a single peak, with retention time of approximately 50.5 min. Flavan-3-ols with DP between 8 and 10 could not be resolved but were presumed to elute between the DP 7 peak and the DP > 10 peak, based on published data [22, 23].

3.2 Effect of flavan-3-ols on OA cell viability

The effects of flavan-3-ol fractions on the viability of two OA cell lines, OE-33 and OE-19, were investigated (Fig. 2). When cells were treated with pure monomers ((−)-epicatechin and (+)-catechin) or dimers (B1, B2) no significant alterations in cell number relative to untreated control were detected under any of the conditions of treatment. The aDP 2.3 fraction had no effect on OE-19 cells at either concentration, but did reduce the OE-33 cell number after 48 h of exposure at 60 mg/L concentration, although not significantly. The concentration of 60 mg/L is equivalent to a total concentration of flavan-3-ol monomers of 200 μM.

Treatment with 60 mg/L of aDP 3.9 reduced OE-33 cell number in a time-dependent manner ($p < 0.0001$). A similar time-dependent effect of 60 mg/L aDP 9.2 was observed in OE-33 and OE-19 cells ($p < 0.0001$ and <0.01, respectively). A comparison of results obtained after treatment of OE-33 cells with apple-derived flavan-3-ol fractions supplemented at 60 mg/L for 48 h showed a positive correlation between aDP and cell number reduction. The reduction in cell number induced by the aDP 9.2 fraction was significantly greater than for the aDP 2.3 and aDP 3.9 ($p < 0.0001$ and <0.05, respectively). Also, the effect of aDP 3.9 was significantly greater than that induced by aDP 2.3 ($p < 0.0001$). The same trend was evident when OE-33 cells were treated with the same fractions at 60 mg/L for 24 h, although the effects were not statistically significant (Fig. 2A). Similarly, treatment of OE-19 cells with aDP 9.2 at 60 mg/L reduced cell number more than aDP 3.9 after 24 and 48 h of treatment, with statistical significance only after 48 h ($p < 0.05$) (Fig. 2B). Both aDP 3.9 and aDP 9.2 fractions decreased cell viability to a significantly greater extent ($p < 0.001$) in OE-33 cells relative to OE-19 cells (Figs. 2A and B).

3.3 Effect of flavan-3-ols on the cell cycle of OA cell lines

OE-33 and OE-19 cells were treated with flavan-3-ol fractions and effects on the cell cycle were investigated using flow cytometry. Pure flavan-3-ol monomers and dimers did not induce any significant alterations in OE-33 or OE-19 cells. Exposure to aDP 3.9 and 9.2 fractions induced similar effects on both cell lines, with accumulation in G0/G1 phase of the cell cycle, accompanied by a reduction of the population of cells in S phase. These responses were not time-dependent. An increase of the population of cells in G0/G1 was also observed in OE-33 cells, but not OE-19 cells, after exposure to the aDP 2.3 fraction (Fig. 3).

3.4 Effect of flavan-3-ols on apoptosis in OA cell lines

The effect of flavan-3-ol fractions on apoptosis in OE-33 and OE-19 cells was investigated using pan-caspase labelling and flow cytometry. Pure flavan-3-ol monomers and dimers did not induce any increase in apoptotic cells in OE-33 or OE-19 cells (Fig. 4). The aDP 2.3 fraction did not induce any effect on OE-19 cells, but a significant increase in the number of apoptotic cells was observed after exposure of OE-33 cells for 48 h. The aDP 3.9 and 9.2 fractions induced apoptosis in a time-dependent manner in OE-33 cells after 24 and 48 h of exposure ($p < 0.05$ and <0.001, respectively). The time-dependent increases in apoptotic

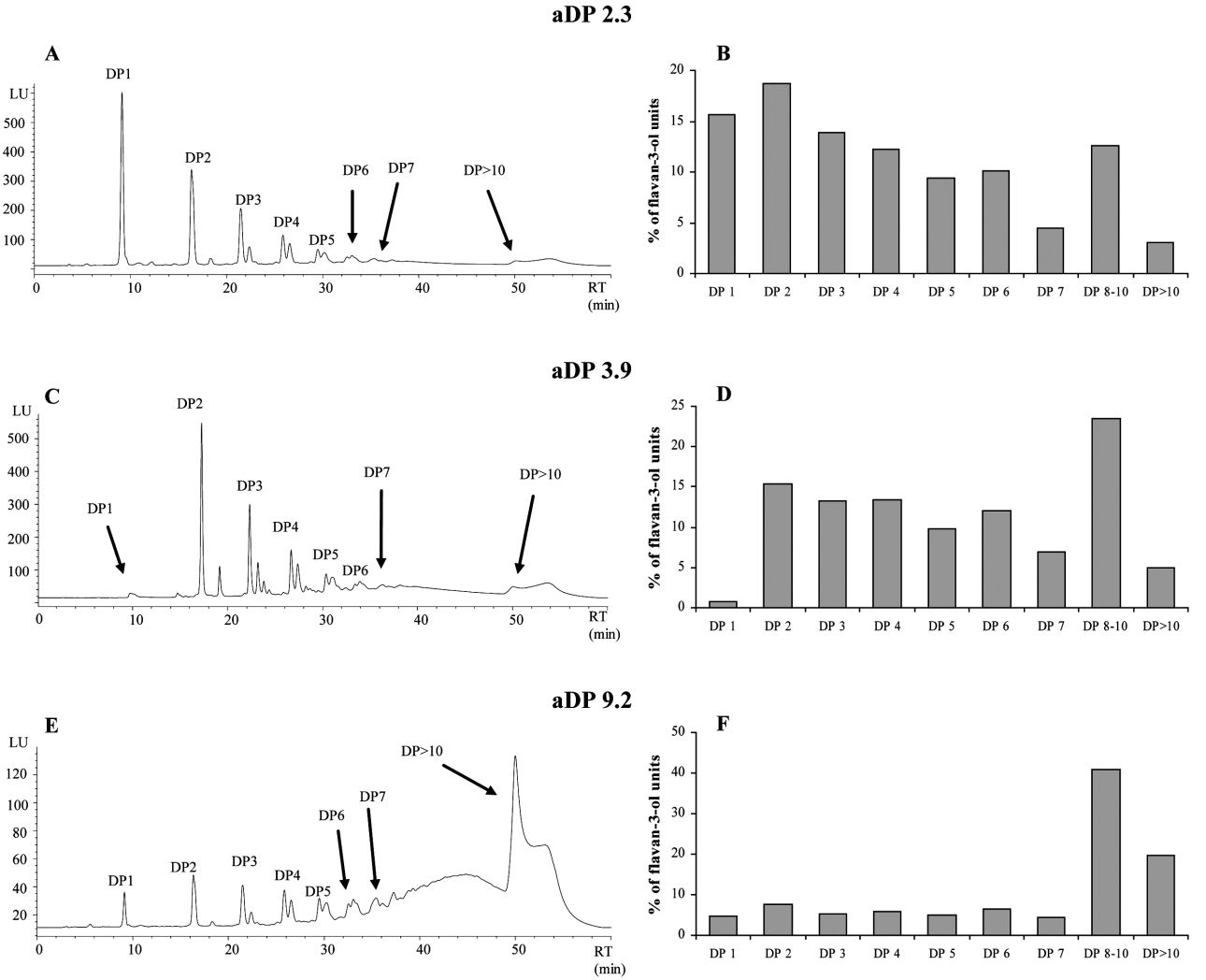

Figure 1. Chromatogram of apple-derived flavan-3-ol fractions: (A) = aDP 2.3 fraction; (C) = aDP 3.9 fraction; (E) = aDP 9.2 fraction. RT, retention time; LU, light intensity units. Estimation of relative abundance of flavan-3-ols with different degrees of polymerization in aDP 2.3 fraction (B), aDP 3.9 fraction (D) and aDP 9.2 fraction. Each value is expressed as a percentage of the total flavan-3-ol units present in the fraction.

events in OE-19 cells after exposure to these two fractions were not statistically significant.

A comparison of the data obtained for the three apple-derived flavan-3-ol fractions and the induction of apoptosis showed a positive correlation between the aDP and the prevalence of apoptotic cells for both cell lines. After 48 h of treatment, the increase in apoptotic events in OE-33 cells by aDP 9.2 was significantly greater than that induced by aDP 3.9 ($p < 0.05$) and aDP 2.3 ($p < 0.001$), while apoptosis induced by aDP 3.9 was significantly greater than that induced by aDP 2.3 ($p < 0.05$) (Fig. 4A). Similarly, the induction of apoptosis in OE-19 cells after exposure to the aDP 9.2 fraction was greater than that induced by aDP 3.9 after 48 h ($p < 0.05$). A similar positive correlation between aDP and apoptosis induction was observed after 24 h exposure, although it was not statistically significant (Fig. 4B). Both aDP 3.9 and 9.2 fractions induced apoptosis to a greater extent (significant in cases of 48 h exposure ($p < 0.001$)) in OE-33 cells compared to OE-19 cells (Figs. 4A and B).

4 Discussion

In the present study, we have investigated the effects of flavan-3-ols on OA cells *in vitro*. Two cell lines were used in order to explore any cell-line or cancer-type specificity in the response to flavan-3-ol treatments. In both lines, exposure of OA cells to two apple-derived flavan-3-ol fractions (aDP 3.9 or 9.2) induced a reduction in cell number that was attributable to an increase in apoptosis and to an arrest of the cell cycle in G0/G1 phase, with a concurrent reduc-

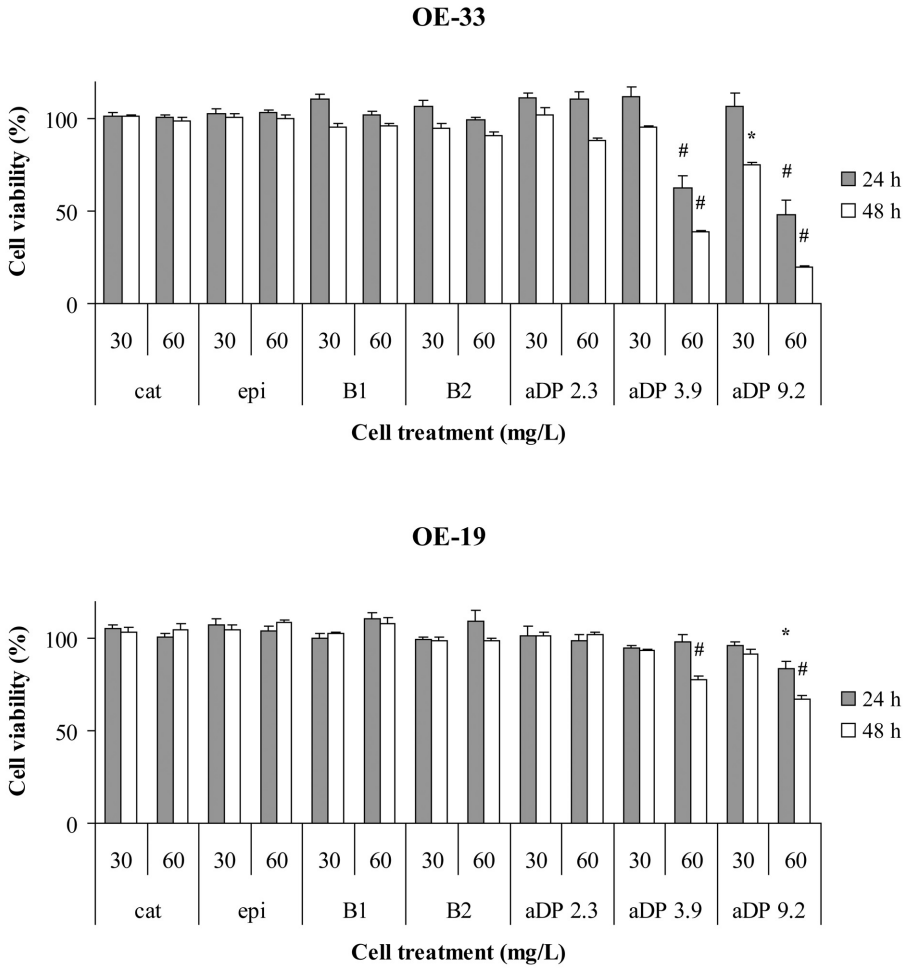

Figure 2. Flavan-3-ol effect on OE-33 (A) and OE19 (B) cell number. Cells were treated with pure flavan-3-ol monomers ((−)-epicatechin and (+)-catechin), dimers (B1 and B2) or apple-derived flavan-3-ol fractions, diluted at 30 or 60 mg/L flavan-3-ol concentration. Cells were treated for 24 or 48 h. Results are expressed as a percentage of viable cells remaining after treatment relative to the untreated control. Data are shown as mean ± SEM ($n = 6$). *, $p < 0.05$; #, $p < 0.0001$.

tion of cells in S phase. These effects were not detected when cells were treated with pure flavan-3-ol monomers or dimers, and the oligomeric fraction with the lower aDP of 2.3 (60% of flavan-3-ol units were in molecules with DP 1–4) only affected one of the cell lines. The increases in apoptosis and reductions in cell number after exposure to flavan-3-ols significantly correlated with the aDP of the flavan-3-ol fractions. This is consistent with the recently reported correlation between the aDP of flavan-3-ol fractions and the reduction in cell viability observed in a colon cancer cell line [16]. Also, one study in a prostate cancer cell line suggested that trimers may have a more pronounced effect than dimers [18]. Other studies using fractioned grape seed extract in various cancer cell lines did not demonstrate any correlation between the degree of polymerization and the magnitude of cellular effects [24, 25]. PA from grape seeds contain flavan-3-ol units that are often galloylated [26]. Gallic acid alone or galloylated flavan-3-ol monomers and dimers have been shown to have significant effects on cell growth and apoptosis induction [17, 18], therefore the lack of a correlation with DP observed previously may be due to the presence and heterogeneous distribution of esterified flavan-3-ol units. The apple-derived procyanidin fractions used in this study consist almost entirely of (−)-epicatechin with <5% of (+)-catechin, with no galloylation. Therefore, the observed effects on cell viability and apoptosis can be attributed solely to the different oligomer/polymer dimensions.

Reductions of cell number after exposure to PA fractions have been reported previously [27]. However, whether these effects occur through extracellular signalling or following the uptake of flavan-3-ols is not known. The aDP 9.2 fraction, which led to the greatest reduction of cell viability and induction of apoptosis at 60 mg/L, consisted mainly of polymers with DP higher than seven (60% of the flavan-3-ol units), suggesting that polymers had greater effect on the cell lines. The high molecular weights of polymeric flavan-3-ols would be expected to limit their ability to penetrate the cell membrane and data reported by Deprez *et al*. [28] supports this notion. Using an *in vitro* model of intestinal absorption, flavan-3-ol monomers, dimers and trimers were shown to be absorbed at similar rates, whereas flavan-3-ols with a higher DP had a ten-fold lower permeability coefficient. The hypothesis that flavan-3-ols might modify cellu-

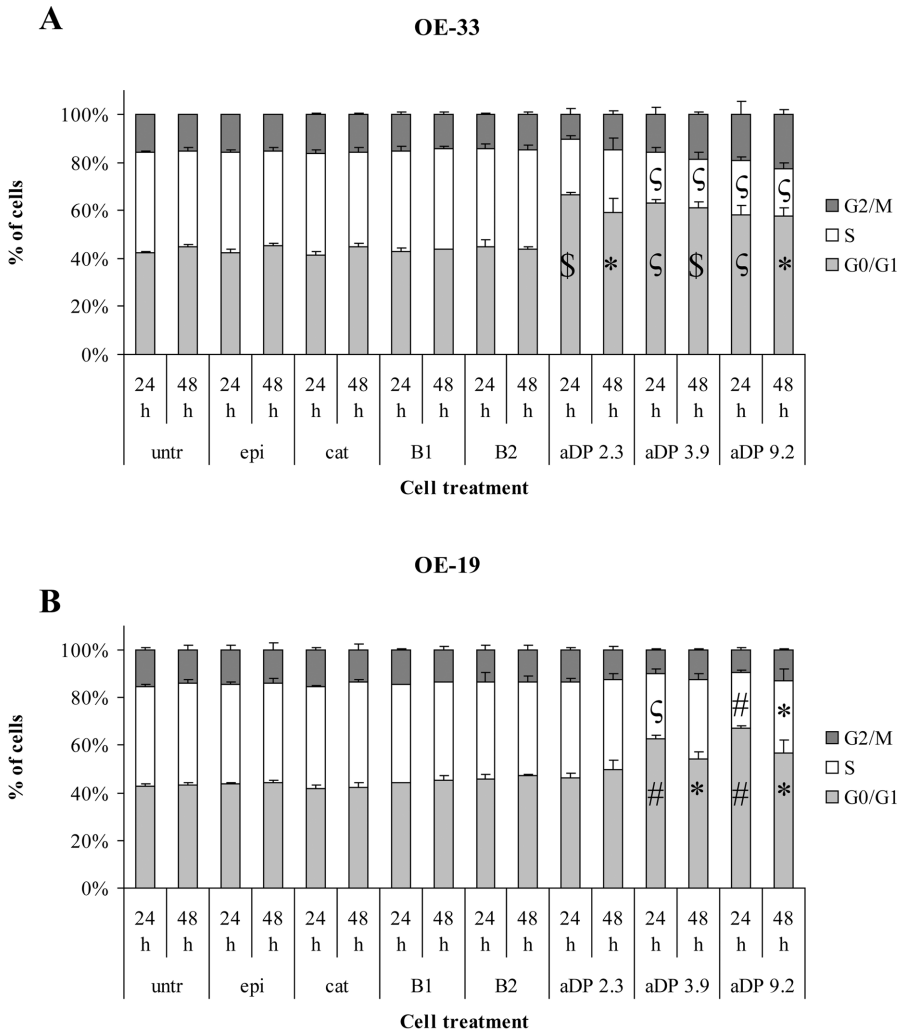

Figure 3. Flavan-3-ol effects on the cell cycle of OE-33 cells (A) and OE-19 cells (B). Results are expressed as a percentage of the cell population in each phase. Data are shown as mean ± SEM ($n = 4$). *, $p < 0.05$; $, $p < 0.01$; ς, $p < 0.001$; #, $p < 0.0001$.

lar functions by interacting with the cell membrane and altering cell signalling pathways has been investigated previously. Activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) and of the c-jun N-terminal kinase 1 and 2 (JNK1/2) signalling cascades has been reported in colon adenocarcinoma cells after exposure to PA [14]. Activation of the JNK1/2 cascade was also observed in prostate cancer cell lines, and in one study this was accompanied by the activation of the p38 mitogen-activated protein kinase (MAPK) pathway and inhibition of the Akt pathway [29, 30]. However, PA were also shown to inhibit the ERK1/2, JNK1/2 and p38 MAPK pathways induced in prostate carcinoma cells by fibroblast-conditioned medium [31]. These conflicting observations suggest further investigation is required to understand which signal cascades are altered by PA treatment and how these alterations are responsible for the observed activation of caspases and alterations to the cell cycle.

In several studies with cell lines derived from various tumour types, previous authors have reported a similar activation of caspases, including caspase-3, following exposure to PA [14, 24, 32], while others reported an arrest of the cell cycle in G0/G1 phase [13, 33]. In contrast, when normal cells were exposed to flavan-3-ols no effects were detected [30, 34], suggesting that flavan-3-ols might only affect transformed cells. In colon cancer cells, the cell cycle arrest in G0/G1 was accompanied by an increased p53-independent expression of p21, along with a reduction of protein levels of cyclin-dependent kinase (CDK) and cyclins involved in cell cycle progression from G0/G1 to S phase [13]. The altered expression of these key regulators of the cell cycle offers a plausible explanation of the molecular mechanism responsible for the cell cycle arrest. However, p21 up-regulation was not observed in *in vitro* and *in vivo* models of prostate cancer, and alterations of the expression of CDK and cyclins were not fully consistent with those observed in colon cancer cells [30, 35]. Therefore, a confirmation of the relevance of these alterations and their role in the mechanism of induction is still needed. In addition to these previous studies demonstrating an arrest of cell cycle in G0/

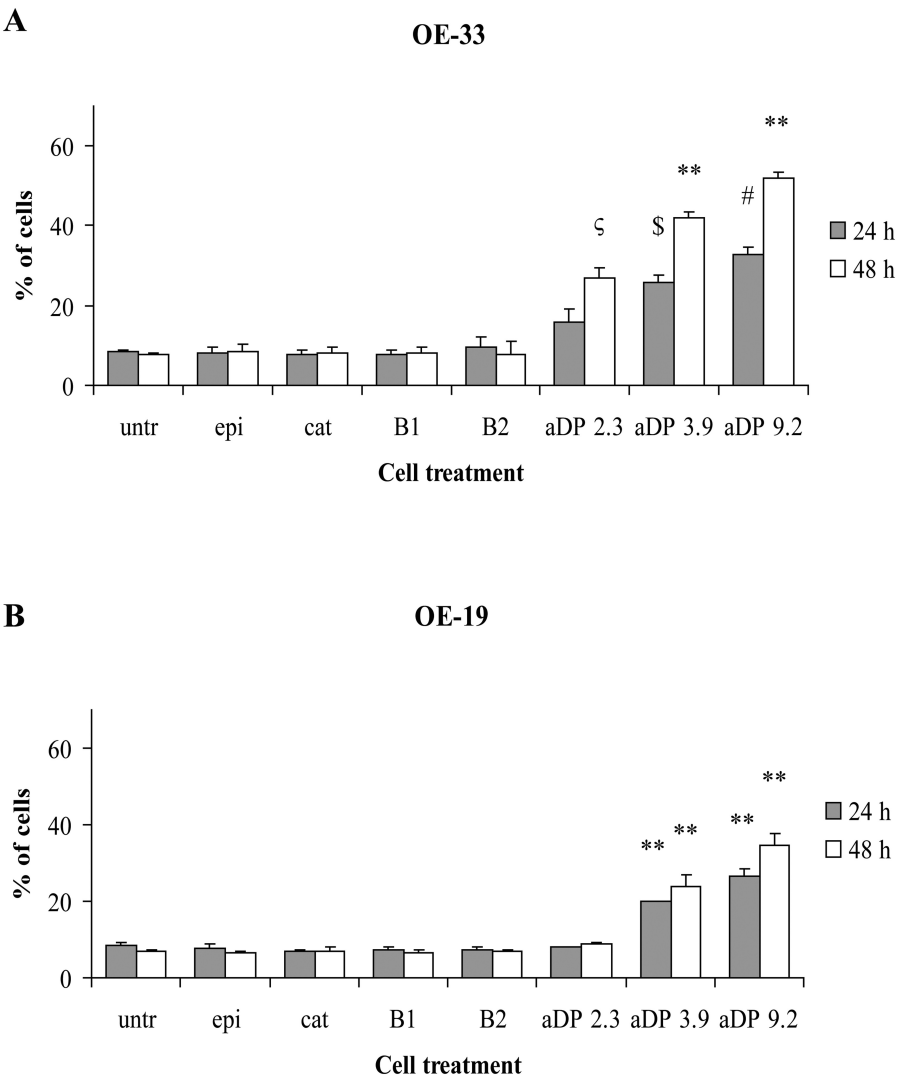

Figure 4. Apoptosis induction after flavan-3-ol exposure in OE-33 cells (A) and OE-19 cells (B). Results are expressed as the number of apoptotic cells as a percentage of the total cell population. Data are shown as mean ± SEM ($n = 4$). \$, $p < 0.01$; ς, $p < 0.001$; #, $p < 0.0001$; **, $p < 0.00001$.

G1, further reports describing studies with different *in vitro* models of colon cancer indicate an arrest of the cell cycle in G2/M phase induced by PA [12, 14]. Surprisingly, exposure of colon adenocarcinoma cells to the aDP 3.9 fraction resulted in an increase proportion of cells in G2/M phase [14]. This suggests that the phase in which the cell cycle is arrested is not dependent on the flavan-3-ol composition of the PA fraction, but it may be due to a tissue specific or cell-type specific response. However, further evidence is required in order to characterize the causes of different responses of the cell cycle to flavan-3-ols.

Our results indicate that procyanidins are less effective at reducing cell viability and at inducing apoptosis in the cell line established from a more advanced stage of cancer progression (OE-19). Although this requires further investigations, these results raise the possibility that one or more signalling cascades becomes increasingly disrupted during cancer progression [36, 37], resulting in a reduced sensitivity to PA exposure.

Data presented here demonstrate for the first time that procyanidins can alter the cell cycle and induce apoptosis in oesophageal cancer cells, with a direct correlation between the magnitude of the effect and the degree of polymerization of the flavan-3-ols applied, and suggesting a mechanism whereby polymeric PA may have the potential to affect the development of OA. Epidemiological studies have shown an inverse correlation between risk of OA and consumption of fruits and vegetables, many of which are rich sources of PA [3 – 5]. A previous *in vivo* study showed that PA reach the small intestine without any alteration to the HPLC profile [38], indicating that PA remain intact in the stomach and would be available to interact with epithelial cells of the oesophagus, not only during ingestion of food, but also during any episodes of reflux of gastric contents into the oesophagus, frequently occurring in patients with gastro-oesophageal reflux disease, which is shown to predispose to OA [39]. Although a precise quantification is still missing, flavan-3-ol intake has been estimated to range

between tens and hundreds of mg/day, widely varying with the type of diet [40]. A more recent investigation suggested that the flavan-3-ol dietary intake might be about 160 mg/day in USA [41]. Concentrations of flavan-3-ol fractions in the oesophageal lumen are therefore potentially high, and the levels used in this *in vitro* study are entirely achievable through diet by consumption of foods rich in flavan-3-ols, such as red fruits, chocolate, red wine or apples. An understanding of how these important dietary components may affect OA, which is an increasingly significant disease in industrialized western societies, requires further investigation, both to elucidate the molecular mechanisms by which flavan-3-ol oligomers and polymers induced their observed effects, and to determine whether similar effects occur *in vivo*.

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The authors have declared no conflict of interest.

5 References

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