Effect of Pectin on the Expression of Proteins Associated with Mitochondrial Biogenesis and Cell Senescence in HT29-Human Colorectal Adenocarcinoma Cells

José Javier Zamorano-León[1], Sandra Ballesteros[2], Natalia de las Heras[2], Luis Alvarez-Sala[3,4], Mariano de la Serna-Soto[3], Khaoula Zekri-Nechar[3], Gala Freixer[3], Bibiana Calvo-Rico[5], Zhengguang Yang[3], José Manuel García-García[5], Vicente Lahera[2], and Antonio José López-Farré[3]

Department of [1]Public Health and Maternal and Child Health, [2]Physiology, and [3]Medicine, School of Medicine, Universidad Complutense de Madrid, Madrid 28040, Spain
[4]Internal Medicine Unit, Hospital General Universitario Gregorio Marañon, Madrid 28007, Spain
[5]Physical Activity and Sport Sciences Department, School of Sport Sciences, Universidad de Castilla-La Mancha, Toledo 13071, Spain

ABSTRACT: Mitochondria dynamic is regulated by different proteins, maintaining a balance between fission and fusion. An imbalance towards mitochondrial fission has been associated with tumor cell proliferation. The aim of this study was to analyze whether pectin modifies the viability of human colon cancer cells and the expression of proteins involved in mitochondrial fusion and fission. The human colon carcinoma cell line HT29 cells was growth in 10% fetal bovine serum in the absence and presence of pectin. Pectin reduced HT29 cell viability in a concentration-dependent manner, reaching a plateau at 150~300 μmol/L pectin. The presence of 200 μmol/L pectin reduced the expression of dynamin-related protein-1 and increased expression of the mitochondrial fusion-associated proteins mitofusin-1 and 2. Expression of cyclin B1, a protein involved in G2/M transition, was found decreased in pectin-incubated HT29 cells. Moreover, expression of p53 protein, the amount of p53 in the nucleous and β-galactosidase activity, which are all biomarkers for cellular senescence, were significantly higher in pectin-incubated HT29 cells than in HT29 cells incubated without pectin. Expression of the protein B-cell lymphoma 2 (Bcl-2) homologous antagonist/killer was increased in response to incubation with pectin. However, incubation with pectin did not affect expression of Bcl-2-associated X protein or Bcl-2, or the caspase-3 activity. Overall, we concluded that pectin reduces the viability of human HT29 colon cancer cells, which is accompanied with a shift in the expression of proteins associated with mitochondrial dynamics towards mitochondrial fusion. Moreover, incubation with pectin favors cellular senescence over apoptosis in HT29 cells.

Keywords: cell proliferation, cellular senescence, colon cancer, mitochondria biogenesis, pectin

INTRODUCTION

Colorectal cancer is one of the deadliest types of cancer and is the most common diet-related cancer. Fiber intake is regularly recommended to decrease the risk of colon cancer (Burkitt, 1971). Indeed, epidemiological studies have shown that increased fruit and vegetable consumption is associated with a reduction in the risk of developing colorectal cancer (Aune et al., 2011; Watson and Collins, 2011).

Pectin is a polysaccharide and source of dietary soluble fiber that is resistant to digestion by human intestinal enzymes, allowing it to reach the colon (Liu et al., 2003). Pectin is a component of fruits such as apples, Canary bananas, citrus fruits, and grapes, and vegetables such as carrots, potatoes, and green beans. Several *in vitro* studies have shown that pectin has anti-tumor properties in different cancer type cells, including melanoma and prostate cancer cells (Han et al., 2006; Jackson et al., 2007). In mice, oral intake of soluble pectin fragments inhibits growth and metastasis of transplanted tumors, including colon tumor (Liu et al., 2008).

Different mechanisms have been suggested to explain the anti-tumor effects of pectin, such as immune-poten-

tiation, anti-mutagenic potential, and the regulation of transformation-related microRNA/oncogenes, amongst others (Chen et al., 2006; Flint et al., 2008; Georgiev et al., 2012; Cheng et al., 2011; Hensel and Meier, 1999). Most of these mechanisms have been directly or indirectly associated with enhancement of the probiotic activity of pectin and/or with inhibition of galectin-3 activity, a multifaceted and pro-metastatic protein which is upregulated in several cancers (Cheng et al., 2011; Schmidgall and Hensel, 2002; Li et al., 2012). However, the mechanisms by which pectin exerts its anti-tumor effects are not fully known.

Mitochondria are crucial organelles for regulating cellular energy generation, calcium and redox homeostasis, and cellular apoptosis. Mitochondria constantly undergo fission and fusion in a dynamic process (Patrushev et al., 2015). Under physiological conditions, the frequencies of fusion and fission events are finely balanced to maintain mitochondrial homeostasis (Patrushev et al., 2015). Mitochondrial fusion and fission are regulated by a number of guanosine triphosphatases (GTPases) proteins (Patrushev et al., 2015; Archer, 2013; Boland et al., 2013). Mitofusins 1 and 2 (Mfn-1 and Mfn-2) mediate mitochondrial fusion while dynamin-related protein-1 (Drp-1) mediates mitochondrial fission (Patrushev et al., 2015; Archer, 2013; Boland et al., 2013). Changes to the balance between the expression of these proteins are frequently observed in cancer cells (Archer, 2013; Boland et al., 2013). Downregulation of Drp-1 promotes mitochondrial fission, decreases cell proliferation, and increases apoptosis of colon cancer cells (Inoue-Yamauchi and Oda, 2012).

Carcinogenesis is associated with a progressive reduction in the ability of cells to trigger apoptosis and senescence, two essential tumor-suppressor mechanisms that are important barrier against cancer progression. Cancer cells develop immortalization by escaping cellular senescence; promoting tumor cell senescence has been proposed as a potential anti-cancer therapy (Nardella et al., 2011). However, apoptosis acutely eliminates potentially harmful cells. Translocation of the tumor suppression protein p53 to the nucleus is thought to be pivotal for stimulating both cellular apoptosis and senescence (Kruiswijk et al., 2015).

Emerging works have suggested the possible contribution of mitochondria dynamics to regulate both apoptosis and cellular senescence (Ziegler et al., 2015; Yoon et al., 2006). In this regard, a close relationship between mitochondrial dynamics and the pro- and anti-apoptotic Bcl-2 family of proteins have been reported; these proteins regulate the release of proteins from the mitochondria into the cytosol, where they stimulate caspase-3 activity (Otera and Mihara, 2012; Dewson and Kluck, 2009). Moreover, senescence has been associated with accumulated dysfunction in mitochondria, resulting in reductions in mitochondrial oxidative phosphorylation (Korolchuk et al., 2017).

Taken all together, despite the knowledge about the modifications of the mitochondrial dynamics associated with cancer, the possible effect of pectin on mitochondrial dynamics remains to be analyzed. Therefore, the aim of the present study was to examine if the presence of pectin may modify growth of human colon cancer cells and if it is associated with changes in the expression of proteins associated with mitochondrial fusion and fission. Moreover, we examined if pectin may modify the expression of proteins associated with either cellular senescence and cellular apoptosis in HT29 cells.

MATERIALS AND METHODS

HT29 colon cancer cells
The human colon carcinoma cell line, HT29 (ATCC, Manassas, VA, USA) were cultured in McCoy's 5A medium (ATCC) containing 16.7 mmol/L glucose, 200 U/mL penicillin, 200 μg/mL streptomycin, and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. Cells were sub-cultured every 2 to 3 days. Before the start of experiments, HT29 cells were synchronized by incubation with 0.5% fetal calf serum (FCS) for 24 h.

All experiments were performed under proliferating conditions by incubating HT29 cells for 24 h at 37°C in the humidified atmosphere of 5% CO_2 in a sub-confluent state (75% confluence and with 10% FBS), in both the absence and presence of pectin (P7536, Sigma-Aldrich Co., St. Louis, MO, USA). This pectin was originally extracted with hot acidic water. Pectin was prepared by diluting 0.06 mg pectin powder in 1 mL McCoy's 5A medium (309 μmol/L final concentration) and heating at 37°C for 30 min. HT29 cells were incubated with pectin for 24 h at 37°C.

At the end of the incubation period, HT29 cells were lysed in a buffer containing 20 mmol/L Tris-HCl, 137 mmol/L NaCl, 10% glycerol, 1% Nonidet-P40, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L ethylenediaminetetraacetic acid, and 2 mmol/L sodium orthovanadate, and were immediately frozen at −80°C until molecular parameters were determined.

Detection of the effect of pectin on HT29 colorectal cancer cell proliferation using XTT assay
HT29 colon cancer cells were seeded onto a 96 well plate at $5×10^3$ cells/well. Twenty four hours after seeding, cells were synchronized by incubation with 0.5% FCS for 24 h. Medium was removed and changed with fresh medium containing 10% FBS with or without pectin (0~300 μmol/L). Following culture for 24 h, proliferation was determined using a commercial cell proliferation kit 3-

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (XTT), following manufacturer's recommendations (Product code: A8088,100; PanReac AppliChem, Barcelona, Spain). The optical density (OD) was measured using a microplate reader at 450 nm. As recommended by the manufacturer, to measure non-specific readings OD were also measured at 650 nm for normalization, and subtracted from the 450 nm measurement.

Cell survival rate was calculated as %={(A sample−A blank)/(A control−A blank)}×100%. The OD of samples were measured after incubation of pectin, and the OD of the control was measured following incubation with 10% FBS without pectin. The blank was the OD of the background.

Western blots analysis
Protein expression was determined by Western blotting. In brief, and as we have previously reported (Zamorano-León et al., 2013; Moñux et al., 2017), protein concentrations were estimated using the bicinchoninic acid reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and equal amount (40 μg/lane) of each lysate were loaded onto denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis 15% (w/v) polyacrylamide gels. The gels were blotted onto nitrocellulose (NC) membranes. As previously reported (Zamorano-León et al., 2013; Moñux et al., 2017), NC membranes were blocked with 5% (w/v) bovine serum albumin (BSA) and incubated with monoclonal antibodies against Mfn-1 (1:2,000, sc-166644, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Mfn-2 (1:1,000, sc-2031, Santa Cruz Biotechnology, Inc.), Drp-1 (1:750, Ab56788, Abcam, Cambridge, UK), and B-cell lymphoma 2 (Bcl-2)-antagonist/killer (Bak) (1:1,000, Ab32371, Abcam), and polyclonal antibodies against p53 (1:1,000, sc-6243, Santa Cruz Biotechnology, Inc.), cyclin B1 (1:1,500, Ab2949, Abcam), Bcl-2-associated X protein (Bax) (1:1,000, sc-493, Santa Cruz Biotechnology, Inc.), and Bcl-2 (1:1,000, sc-783, Santa Cruz Biotechnology, Inc.). A monoclonal anti-β-actin antibody (1:7,500, A-5441, Sigma-Aldrich Co.) was used as a control for protein loading. NC membranes were then incubated with peroxidase-conjugated anti-mouse IgG (1:2,000 for Mfn-1 and Drp-1, 1:1,000 for Mfn-2, and 1:2,500 for β-actin) and peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:2,500 for p53, Bak, Bax, and Bcl-2). The protein signal was obtained using chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK) that was detected and densitometrically analysed using an Ibright Imaging System (Ibright FL100, Thermo Fisher Scientific, Inc.).

β-Galactosidase, cytochrome C oxidase, and caspase-3 activities
Following incubation of HT29 cells with and without 200 μmol/L pectin. β-Galactosidase activity was determined at 405 nm using a commercial assay kit following manufacturer's recommendations (75707, Thermo Fisher Scientific, Inc.). β-Galactosidase activity in cells incubated with pectin was calculated as a percent of the activity in cells incubated without pectin.

Cytochrome C oxidase activity was determined in HT29 cells incubated with and without 200 μmol/L pectin using a commercial kit (ab10909. Abcam). The kit was carried out following manufacturer's recommendations. Cytochrome C oxidase activity was determined at 550 nm. The intra-assay and inter-assay variabilities were <7% and <10%, respectively.

Caspase-3 activity was measured using a colorimetric commercial kit (ab39401, Abcam) based on the detection of the chromophore p-nitroaniline. In accordance to manufacturer's specifications, caspase-3 activity was expressed as OD units at 405 nm.

Determination of the nuclear content of p53
Nuclear extracts from HT29 cells incubated in the presence and absence of pectin were obtained according to the method described by Schreiber et al. (1990). Samples were centrifuged at 12,000 g at 4°C for 10 min, followed by an additional centrifugation at 12,000 g for 15 min at 4°C. The nuclear extracts were immediately lysed and protein concentration was measured using the bicinchoninic acid reagent.

The amount of p53 content in the cellular nucleus was determined by dot-blot technique. Due to the relative low level of nuclear protein obtained, it was not possible to use Western blotting to determine nuclear p53 contents. As previously reported, dot blot analysis was carried out through loading 5 μg of total nuclear protein onto NC membranes (López-Farré et al., 2012; Ortega et al., 2007). As with the Western-blot analysis, NC membranes were blocked with 5% BSA (w/v), washed twice, and were incubated with the polyclonal p53 primary antibody followed by the peroxidase-conjugated anti-rabbit IgG secondary antibody above described. The protein signal was detected by chemiluminiscence.

Statistical analysis
Values are expressed as mean±standard error of mean (SEM). Unless it is otherwise indicated, six experimental repeats were performed for each determined variable. Mann-Whitney's test was used to compare differences between groups. A P value <0.05 was considered statistically significant. The statistical analysis was performed using the software SPSS version 22.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Effect of pectin on HT29 cells viability

HT29 cells viability, when considering cell viability as an index of cell proliferation, was significantly increased McCoy's 5A medium was supplemented with 10% FBS (Fig. 1). Addition of pectin to 10% FBS significantly reduced HT29 cell viability (Fig. 1A). The effect of pectin was concentration dependent and maximum inhibition was observed with pectin concentrations over 150 μmol/L (Fig. 1B). The most homogenous inhibition of HT29 cells viability was observed with 200 μmol/L pectin (showing

the lowest standard error). Therefore, 200 μmol/L pectin was used during subsequent experiments.

The effect of pectin on cell viability was not dependent on a cellular cytotoxic effect, since trypan blue exclusion was similar between HT29 cells incubated in the presence and in the absence of pectin (data not shown).

Changes in proteins involved in mitochondrial dynamics and in mitochondrial functionality

Through Western blot, we demonstrated that HT29 cells incubated with 200 μmol/L pectin had lower expression of Drp-1 protein than cells incubated in the absence of
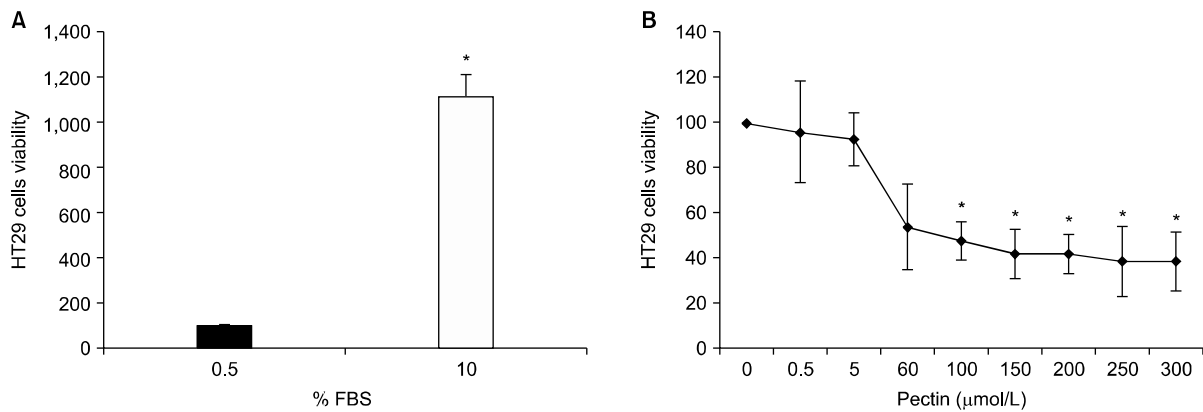

Fig. 1. (A) The viability index of HT29 cells incubated in culture medium containing 0.5% or 10% fetal bovine serum (FBS). Results are represented as mean±SEM of three experimental repeats. *P<0.05 with respect to 0.5% FBS. (B) Effects of increased pectin concentrations on HT29 cell viability. Results are expressed as mean±SEM of six experimental repeats. *P<0.05 with respect to HT29 incubated without pectin.
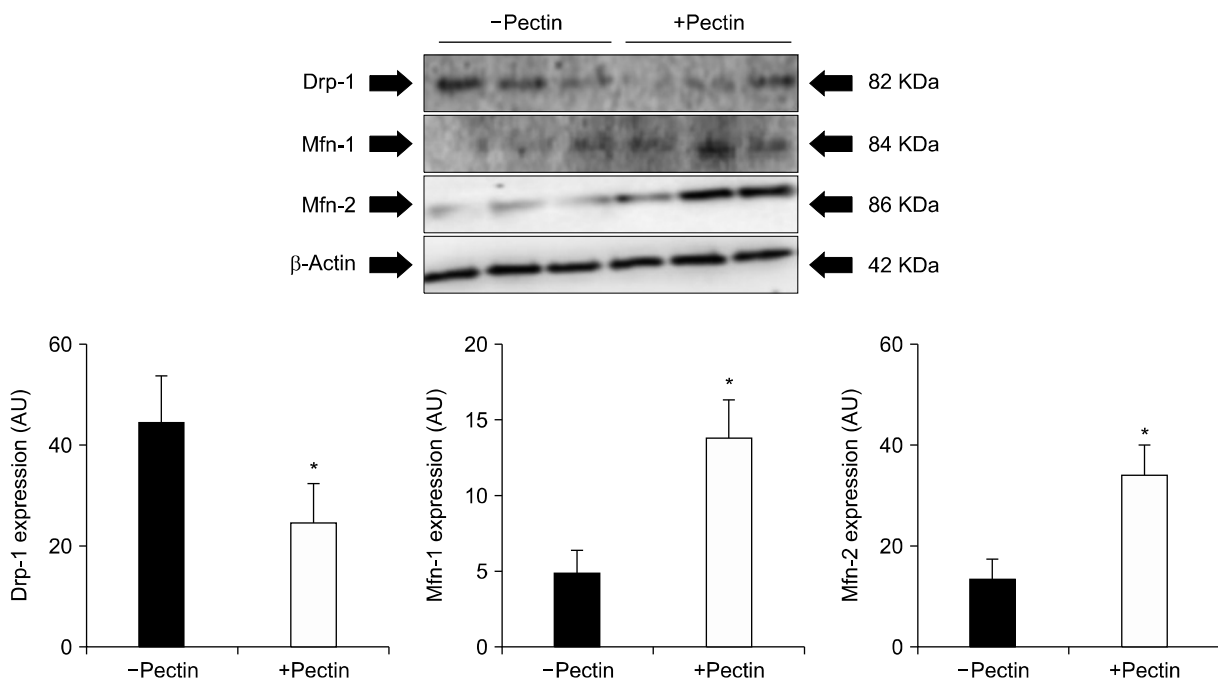

Fig. 2. Representative Western blots showing the expression of proteins associated with mitochondrial fission, dynamin-related protein 1 (Drp-1), and mitochondrial fusion, mitofusin (Mfn)-1 and Mfn-2 in HT29 cells incubated with and without pectin (200 μmol/L). The expression of β-actin was used as loading control. Bar graphs show the densitometric analysis of all the Western blots in arbitrary units (AU). Results are presented as mean±SEM of six experimental repeats. *P<0.05 with respect to HT29 cells incubated without pectin.

pectin (Fig. 2). Moreover, HT29 cells incubated with pectin showed higher expression of Mfn-1 and Mfn-2 than HT29 cells incubated without pectin (Fig. 2).

The activity of cytochrome C oxidase, a protein included in the mitochondrial phosphorylation chain, was significantly reduced in HT29 cells incubated with pectin compared to those incubated without pectin (Fig. 3).

Effect of pectin on expression of proteins related to cellular senescence

β-Galactosidase activity was significantly higher in HT29

cells incubated with 200 μmol/L pectin than in the cells incubated without pectin (% β-galactosidase activity increased: $9.95 \pm 3.22\%$, corrected to μg protein, n=4; $P<$ 0.05). Significantly higher expression of total p53 protein was also observed in HT29 cells incubated with pectin than in the cells incubated without pectin (Fig. 4). Moreover, the nuclear content of p53 protein was also significantly higher in following incubation with pectin than in the nucleus of HT29 cells incubated without pectin (Fig. 4). In addition, we observed a significant reduction in cyclin B1 expression in cells incubated in 200 μmol/L pectin compared to those incubated without pectin (Fig. 4).

Expression and activity of apoptotic-associated proteins

HT29 cells incubated with 200 μmol/L pectin showed increased amounts of the pro-apoptotic protein Bak compared to HT29 cells incubated without pectin (Fig. 5). However, the cellular content of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 was not different between the HT29 cells incubated in the presence and in the absence of pectin (Fig. 5). Moreover, caspase-3 activity did not show significant differences between HT29 cells incubated with or without pectin (Fig. 6).
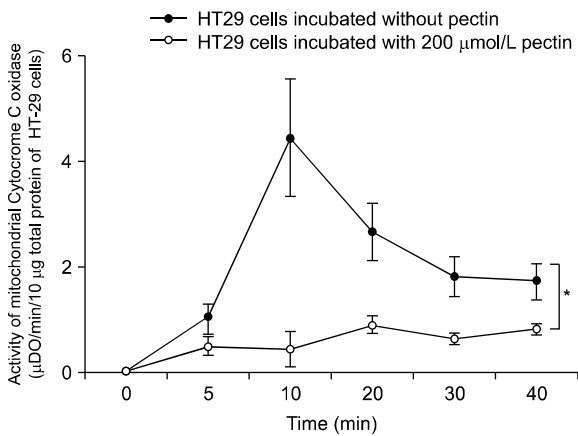
DISCUSSION

The study is, to our knowledge, the first examining the role of pectin on the expression of proteins associated

Fig. 3. Activity of mitochondrial citocrome C oxidase in HT29 cells incubated with and without pectin (200 μmol/L). Results are presented as mean±SEM of six experimental repeats. *$P<$ 0.05 between curves.
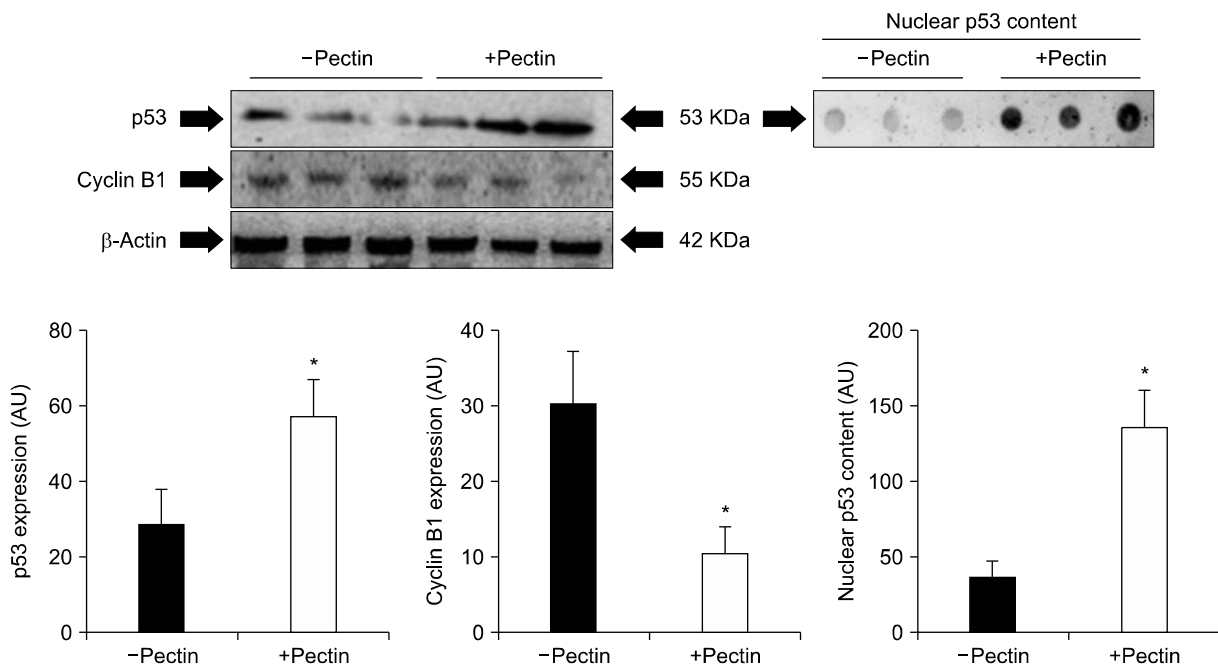

Fig. 4. Representative Western blots of the expression of proteins associated with cellular senescence, p53 and cyclin B1, in HT29 cells incubated with and without pectin (200 μmol/L). The expression of β-actin was used as a loading control. Representative dot blots showing the content of nuclear p53 in HT29 cells incubated with and without pectin (200 μmol/L). Bar graphs show the densitometric analysis of all Western blots and dot blots in arbitrary units (AU). Results are presented as mean±SEM of six experimental repeats. *$P<0.05$ with respect to HT29 cells incubated without pectin.
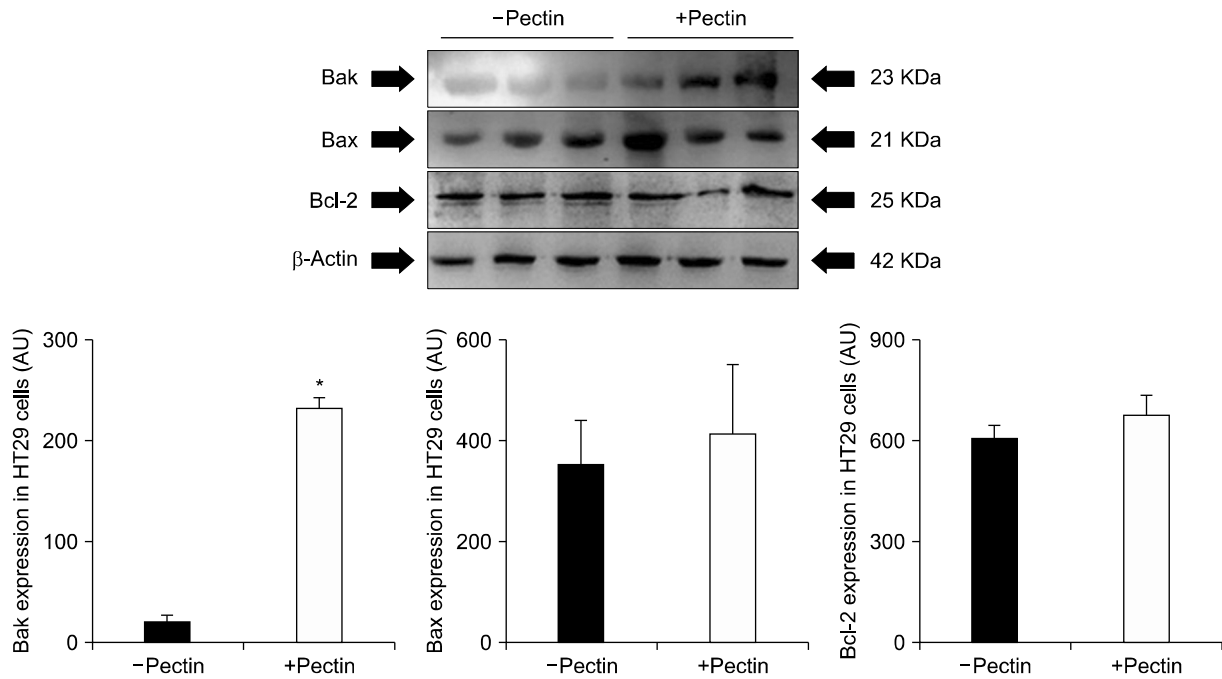
Fig. 5. Representative Western blots to determine the expression of proteins associated with cellular apoptosis, B-cell lymphoma 2 (Bcl-2)-antagonist/killer (Bak), Bcl-2-associated X protein, and Bcl-2, in HT29 cells incubated with and without pectin (200 μmol/L). The expression of β-actin was used as loading control. Bar graphs show the densitometric analysis of all the Western blots in arbitrary units (AU). Results are presented as mean±SEM of six experimental repeats. *$P<0.05$ with respect to HT29 cells incubated without pectin.
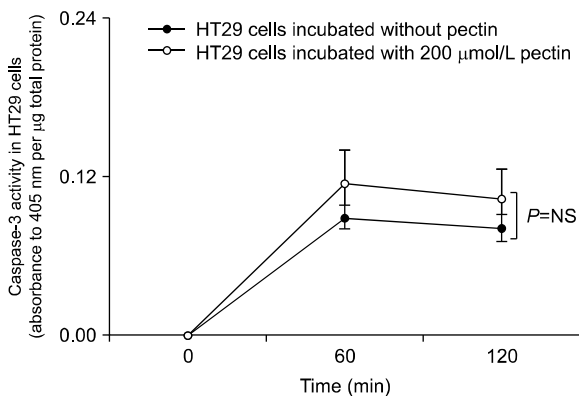

Fig. 6. Caspase-3 activity was determined in HT29 cells incubated with and without pectin (200 μmol/L). Results are presented as mean±SEM of six experimental repeats. NS: not significant.

with mitochondrial fusion and fission in colorectal human colorectal HT29 cancer cells. Changes in expression were accompanied by a reduction in HT29 cell viability. Moreover, incubation with pectin favoured cellular senescence over apoptosis.

Previous studies have demonstrated that animals treated with azoxymethane or methylnitrosourea develop less colon tumors when their diets were enriched with pectin (Watanabe et al., 1979). Pectin, as dietary fiber, is not enzymatically digested in the small intestine but is degraded by microbia in the colon. Therefore, most of the effects associated with pectin have been attributed to its possible enhancement of probiotic actions (Odun-Ayo et

al., 2015). In this regard, pectin reduced the activity of β-glucuronidase, an enzyme found in fecal bacteria whose activity is correlated to colon cancer development (Ohkami et al., 1995; Lindop et al., 1985). Several studies have also shown that oral consumption of pectin decreases the risk of intestinal infection and diarrhea in children by promoting growth of "good" bacteria in the colon (e.g., *Biffidobacteria* and *Lactobacillus*) and the detriment of pathogenic bacteria (Olano-Martin et al., 2002). However, it should be noted that much less is known about other effects of pectin on colon cells. Our results showed that in cultured human colorectal cancer cells HT29, pectin reduced cellular viability but without inducing cytotoxicity.

As mentioned above, mitochondria maintain a dynamic equilibrium between mitochondrial fission and fusion that is controlled by specific proteins (Patrushev et al., 2015; Archer, 2013; Boland et al., 2013). While both Mfn-1 and Mfn-2 promote mitochondrial fusion, Drp-1 favors mitochondrial fission (Patrushev et al., 2015; Archer, 2013). In several human cancer cells, it was demonstrated that an imbalance between Drp-1/Mfns expression facilitates the mitochondrial fission state. In this regard, increased fission due to high Drp-1 expression and decreased fusion due to loss of Mfns have been linked to cancer cell migration, invasion, and metastasis (Zhao et al., 2013; Ferreira-da-Silva et al., 2015). Moreover, Drp-1 inhibition or knockdown results in a marked reduction in cancer cell proliferation, including of colon cancer cells

(Inoue-Yamauchi and Oda, 2012; Rehman et al., 2012).

In our study, HT29 cells incubated with pectin showed lower expression of Drp-1 and higher expression of both Mfn-1 and Mfn-2 than HT29 cells incubated without pectin. These results suggest that in HT29 cells, pectin promoted a shift by reducing expression of Drp-1 associated with mitochondrial fission, and increasing expression of proteins mediating mitochondrial fusion. This was accompanied by a reduction in HT29 cell viability. In this regard, several studies have demonstrated the relationship between tumor cell proliferation and mitochondrial fission (Rehman et al., 2012; Xie et al., 2015). Zhan et al. (2016) demonstrated that in hepatocellular carcinoma cells, Drp-1-mediating mitochondrial fission promotes cell cycle progression from G1 to S phase, and subsequent cell proliferation (Zhan et al., 2016).

Cellular senescence, broadly defined as the physiological program of terminal growth arrest, is associated with aging. However, cellular senescence is also an important tumor suppressor mechanism (Campisi, 2001). In the present study, under proliferating conditions, the activity of the senescence-associated enzyme β-galactosidase was increased in HT29 cells incubated with pectin compared with HT29 cells incubated without pectin. Moreover, the expression of cyclin B1 was markedly reduced by pectin. Cyclin B1 is crucial for G2/M transition. Increases expression of cyclin B1 has been reported in cancer cells and insufficient levels of cyclin B-Cdk1 complexes, i.e. as a result of attenuation of cyclin B1, are related to cell cycle arrest and reduction of cell proliferation (Innocente et al., 1999). Growth arrest of cells entering senescence may start with p53 activation. In HT29 cells incubated with pectin, p53 expression levels were significantly higher than in cells incubated without pectin. The nuclear content of p53, which is suggestive of p53 translocation from the cytosol to the nucleus, was also increased in HT29 cells incubated with pectin. It has been reported that the level and/or activity of p53 was increased in senescent cells and overexpression of p53 was sufficient to induce cellular senescence in p53-null cells (Serrano et al., 1997; Sugrue et al., 1997). Taken together, these results could suggest that pectin may promote a change in the human carcinoma HT29 cells from a proliferating phenotype to a senescence phenotype. In support of this hypothesis, blockage of mitochondrial fission has been reported to promote the maintenance of elongated mitochondria, which leads to cellular senescence. This suggests that senescence cells are typically associated with an overall shift toward mitochondrial fusion (Jendrach et al., 2005; Lee et al., 2007). Accordingly, during senescence, the fission-associated protein, fission-1 protein, is down-regulated, which leads to mitochondrial elongation (Lee et al., 2007), and inhibition of Drp-1 promotes cellular senescence (Yoon et al., 2006).

Our results showed that in pectin-incubated HT29 cells both senescence and mitochondrial fusion-related proteins were increased while the mitochondrial fission-associated protein, Drp-1, was decreased. In accordance to our observations, several compounds found in fruits and vegetables, such as resveratrol, bisdemethoxycurcumin, baicalin, or ginsenoside, show anti-proliferative effects on different types of cancerous cells, including human colon cancer cells, through induction of cellular senescence (Luo et al., 2013; Li et al., 2013; Wang et al., 2018; Sin et al., 2012).

Despite the above observations, it should be noted that some authors have postulated that HT29 cells contain a mutated p53 isoform that produces a sub-functional version of p53 (Zhou et al., in press). However, the finding that β-galactosidase activity was significantly higher in HT29 cells incubated with pectin strongly supports that pectin promoted HT29 cell senescence. Moreover, other studies have reported induction of HT29 cell apoptosis via p53 activation (Bagheri et al., 2018; Song et al., 2005). With the present study we could not assess whether promotion of HT29 cell senescence by pectin was really mediated by a p53-dependent pathway. Additional experiments are warrant to identify the mechanisms involved in stimulation of β-galactosidase activity by pectin in proliferating HT29 cells.

Another important mechanism mediating tumor cell growth is apoptosis. In these experiments, expression of the pro-apoptotic protein Bak, but not of the pro-apoptotic protein Bax nor the anti-apoptotic protein Bcl-2 was changed by pectin. In addition, caspase-3 activity was not changed by the presence of pectin. Taken together, these results suggest that the apoptotic pathway was not significantly stimulated by pectin in HT29 cells. In this regard, several authors have suggested that mitochondrial fusion is associated with cellular senescence whereas mitochondrial fission is closely related to cellular apoptosis (Lee et al., 2007; Youle and Karbowski, 2005). Interestingly, it has also been demonstrated that chemical inhibitors of mitochondrial chain phosphorylation complexes and aerobic glycolysis induce cellular senescence, and therefore promote cell proliferation arrest (Moiseeva et al., 2009; Wang et al., 2003). Our experiments showed that cytochrome C oxidase activity was significantly lower in HT29 cells incubated with pectin than in HT29 incubated without pectin, suggestive of a reduction in mitochondrial oxidative phosphorylation mediated by pectin. Accordingly, that cytochrome C oxidase downregulation was also recorded in senescent porcine pulmonary artery endothelial cells (Zhang et al., 2002). However, since mitochondrial fusion is mostly associated with increased oxidative phosphorylation (Yao et al., 2019), it was questioned why mitochondrial fusion is promoted by pectin but cytochrome C oxidase activity is downreg-

ulated. Reduction of cytochrome C oxidase may suggest the existence of mitochondrial dysfunctionality. Therefore, it can be speculated that pectin promotes mitochondrial dysfunction through modifying mitochondrial mitophagy, which can cause an increase in the number of dysfunctional mitochondria. Further experiments are needed to assess this hypothesis.

Study limitations

The study has several limitations. The free radical theory of senescence suggests that cellular senescence is associated with higher production of reactive oxygen species (ROS). Since mitochondria are important source of ROS generation, it has been proposed that excessive mitochondrial ROS is important for establishing cellular senescence. In several cell types, increased ROS production has been correlated with increased mitochondrial fission rather than to mitochondrial fusion (Yu et al., 2006). However, cytochrome C oxidative dysfunction has also been associated with increased oxidative stress (Srinivasan and Avadhani, 2013). On the contrary, other studies have suggested that mitochondrial ROS generation may not be the primary cause of cellular senescence. In this regard, it has also been suggested that increased mitochondrial ROS production in replicative senescent cells is a consequence of the senescence phenotype, rather than the cause (Ziegler et al., 2015; Lawless et al., 2012). Therefore, further studies are needed to establish the effects of pectin on HT29 senescence, changes in the mitochondrial fusion and fission balance, and in mitochondrial metabolism and ROS production.

Another major limitation of this study is that we did not determine the molecular mechanisms associated with the changes elicited by pectin on both the expression of proteins related to mitochondrial fusion and fission and on cellular senescence. Moreover, the experimental design did not allow us to establish whether the changes induced by pectin on the mitochondrial dynamics-associated proteins promoted cellular senescence, or if pectin induced changes to proteins associated with cellular senescence, and these favoured a shift in the homeostatic balance mediating mitochondrial fusion and fission. Although the relationship between cellular senescence and mitochondrial dynamics is probably bidirectional, further studies are warranted to understand further.

The results of this study cannot discard the possibility that pectin also promoted apoptosis in HT29 cells. In this regard, pectin stimulated Bak protein expression, however did not induce changes to caspase-3 activity. However, it is possible that pectin induced HT29 cell apoptosis through other mechanisms that are not dependent on caspase-3 activity. Additional studies are needed to analyze this further.

In conclusion, this study suggests that pectin directly impacts the expression of proteins regulating mitochondrial fusion and fission in the human colon carcinoma cell line HT29. Pectin addition was accompanied by a reduction in HT29 cell viability, and stimulation of proteins associated with cellular senescence.

ACKNOWLEDGEMENTS

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

REFERENCES

Archer SL. Mitochondrial dynamics — mitochondrial fission and fusion in human diseases. N Engl J Med. 2013. 369:2236-2251.

Aune D, Lau R, Chan DS, Vieira R, Greenwood DC, Kampman E, et al. Nonlinear reduction in risk for colorectal cancer by fruit and vegetable intake based on meta-analysis of prospective studies. Gastroenterology. 2011. 141:106-118.

Bagheri E, Hajiaghaalipour F, Nyamathulla S, Salehen N. The apoptotic effects of *Brucea javanica* fruit extract against HT29 cells associated with p53 upregulation and inhibition of NF-κB translocation. Drug Des Devel Ther. 2018. 12:657-671.

Boland ML, Chourasia AH, Macleod KF. Mitochondrial dysfunction in cancer. Front Oncol. 2013. 3:292.

Burkitt DP. Epidemiology of cancer of the colon and rectum. Cancer. 1971. 28:3-13.

Campisi J. Cellular senescence as a tumor-suppressor mechanism. Trends Cell Biol. 2001. 11:S27-S31.

Chen CH, Sheu MT, Chen TF, Wang YC, Hou WC, Liu DZ, et al. Suppression of endotoxin-induced proinflammatory responses by citrus pectin through blocking LPS signaling pathways. Biochem Pharmacol. 2006. 72:1001-1009.

Cheng H, Li S, Fan Y, Gao X, Hao M, Wang J, et al. Comparative studies of the antiproliferative effects of ginseng polysaccharides on HT-29 human colon cancer cells. Med Oncol. 2011. 28:175-181.

Dewson G, Kluck RM. Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. J Cell Sci. 2009. 122:2801-2808.

Ferreira-da-Silva A, Valacca C, Rios E, Pópulo H, Soares P, Sobrinho-Simões M, et al. Mitochondrial dynamics protein Drp1 is overexpressed in oncocytic thyroid tumors and regulates cancer cell migration. PLoS One. 2015. 10:e0122308.

Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Microbiol. 2008. 6:121-131.

Georgiev Y, Ognyanov M, Yanakieva I, Kussovski V, Kratchanova

M. Isolation, characterization and modification of citrus pectins. J BioSci Biotech. 2012. 1:223-233.

Han SB, Lee CW, Kang MR, Yoon YD, Kang JS, Lee KH, et al. Pectic polysaccharide isolated from *Angelica gigas* Nakai inhibits melanoma cell metastasis and growth by directly preventing cell adhesion and activating host immune functions. Cancer Lett. 2006. 243:264-273.

Hensel A, Meier K. Pectins and xyloglucans exhibit antimutagenic activities against nitroaromatic compounds. Planta Med. 1999. 65:395-399.

Innocente SA, Abrahamson JLA, Cogswell JP, Lee JM. p53 regulates a G_2 checkpoint through cyclin B1. Proc Natl Acad Sci USA. 1999. 96:2147-2152.

Inoue-Yamauchi A, Oda H. Depletion of mitochondrial fission factor DRP1 causes increased apoptosis in human colon cancer cells. Biochem Biophys Res Commun. 2012. 421:81-85.

Jackson CL, Dreaden TM, Theobald LK, Tran NM, Beal TL, Eid M, et al. Pectin induces apoptosis in human prostate cancer cells: correlation of apoptotic function with pectin structure. Glycobiology. 2007. 17:805-819.

Jendrach M, Pohl S, Vöth M, Kowald A, Hammerstein P, Bereiter-Hahn J. Morpho-dynamic changes of mitochondria during ageing of human endothelial cells. Mech Ageing Dev. 2005. 126: 813-821.

Korolchuk VI, Miwa S, Carroll B, von Zglinicki T. Mitochondria in cell senescence: is mitophagy the weakest link?. EBioMedicine. 2017. 21:7-13.

Kruiswijk F, Labuschagne CF, Vousden KH. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. Nat Rev Mol Cell Biol. 2015. 16:393-405.

Lawless C, Jurk D, Gillespie CS, Shanley D, Saretzki G, von Zglinicki T, et al. A stochastic step model of replicative senescence explains ROS production rate in ageing cell populations. PLoS One. 2012. 7:e32117.

Lee S, Jeong SY, Lim WC, Kim S, Park YY, Sun X, et al. Mitochondrial fission and fusion mediators, hFis1 and OPA1, modulate cellular senescence. J Biol Chem. 2007. 282:22977-22983.

Li Y, Liu L, Niu Y, Feng J, Sun Y, Kong X, et al. Modified apple polysaccharide prevents against tumorigenesis in a mouse model of colitis-associated colon cancer: role of galectin-3 and apoptosis in cancer prevention. Eur J Nutr. 2012. 51:107-117.

Li YB, Gao JL, Zhong ZF, Hoi PM, Lee SM, Wang YT. Bisdemethoxycurcumin suppresses MCF-7 cells proliferation by inducing ROS accumulation and modulating senescence-related pathways. Pharmacol Rep. 2013. 65:700-709.

Lindop R, Tasman-Jones C, Thomsen LL, Lee SP. Cellulose and pectin alter intestinal beta-glucuronidase (EC 3.2.1.31) in the rat. Br J Nutr. 1985. 54:21-26.

Liu HY, Huang ZL, Yang GH, Lu WQ, Yu NR. Inhibitory effect of modified citrus pectin on liver metastases in a mouse colon cancer model. World J Gastroenterol. 2008. 14:7386-7391.

Liu LS, Fishman ML, Kost J, Hicks KB. Pectin-based systems for colon-specific drug delivery via oral route. Biomaterials. 2003. 24:3333-3343.

López-Farré AJ, Zamorano-León JJ, Segura A, Mateos-Cáceres PJ, Modrego J, Rodríguez-Sierra P, et al. Plasma desmoplakin I biomarker of vascular recurrence after ischemic stroke. J Neurochem. 2012. 121:314-325.

Luo H, Yang A, Schulte BA, Wargovich MJ, Wang GY. Resveratrol induces premature senescence in lung cancer cells via ROS-mediated DNA damage. PLoS One. 2013. 8:e60065.

Moiseeva O, Bourdeau V, Roux A, Deschênes-Simard X, Ferbeyre G. Mitochondrial dysfunction contributes to oncogene-induced senescence. Mol Cell Biol. 2009. 29:4495-4507.

Moñux G, Zamorano-León JJ, Marqués P, Sopeña B, García-García JM, Laich de Koller G, et al. FXa inhibition by rivaroxaban modifies mechanisms associated with the pathogenesis of human abdominal aortic aneurysms. Br J Clin Pharmacol. 2017. 83:2661-2670.

Nardella C, Clohessy JG, Alimonti A, Pandolfi PP. Pro-senescence therapy for cancer treatment. Nat Rev Cancer. 2011. 11:503-511.

Odun-Ayo F, Mellem J, Naicker T, Reddy L. Chemoprevention of azoxymethane-induced colonic carcinogenesis in Balb/c mice using a modified pectin alginate probiotic. Anticancer Res. 2015. 35:4765-4775.

Ohkami H, Tazawa K, Yamashita I, Shimizu T, Murai K, Kobashi K, et al. Effects of apple pectin on fecal bacterial enzymes in azoxymethane-induced rat colon carcinogenesis. Jpn J Cancer Res. 1995. 86:523-529.

Olano-Martin E, Gibson GR, Rastell RA. Comparison of the *in vitro* bifidogenic properties of pectins and pectic-oligosaccharides. J Appl Microbiol. 2002. 93:505-511.

Ortega A, Pérez de Prada MT, Mateos-Cáceres PJ, Ramos Mozo P, González-Armengol JJ, González Del Castillo JM, et al. Effect of parathyroid-hormone-related protein on human platelet activation. Clin Sci. 2007. 113:319-327.

Otera H, Mihara K. Mitochondrial dynamics: functional link with apoptosis. Int J Cell Biol. 2012. 2012:821676.

Patrushev MV, Mazunin IO, Vinogradova EN, Kamenski PA. Mitochondrial fission and fusion. Biochemistry. 2015. 80:1457-1464.

Rehman J, Zhang HJ, Toth PT, Zhang Y, Marsboom G, Hong Z, et al. Inhibition of mitochondrial fission prevents cell cycle progression in lung cancer. FASEB J. 2012. 26:2175-2186.

Schmidgall J, Hensel A. Bioadhesive properties of polygalacturonides against colonic epithelial membranes. Int J Biol Macromol. 2002. 30:217-225.

Schreiber E, Harshman K, Kemler I, Malipiero U, Schaffner W, Fontana A. Astrocytes and glioblastoma cells express novel octamer-DNA binding proteins distinct from the ubiquitous Oct-1 and B cell type Oct-2 proteins. Nucleic Acids Res. 1990. 18:5495-5503.

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16[INK4a]. Cell. 1997. 88:593-602.

Sin S, Kim SY, Kim SS. Chronic treatment with ginsenoside Rg3 induces Akt-dependent senescence in human glioma cells. Int J Oncol. 2012. 41:1669-1674.

Song G, Mao YB, Cai QF, Yao LM, Ouyang GL, Bao SD. Curcumin induces human HT-29 colon adenocarcinoma cell apoptosis by activating p53 and regulating apoptosis-related protein expression. Braz J Med Biol Res. 2005. 38:1791-1798.

Srinivasan S, Avadhani NG. Cytochrome c oxidase dysfunction in oxidative stress. Free Radic Biol Med. 2013. 53:1252-1263.

Sugrue MM, Shin DY, Lee SW, Aaronson SA. Wild-type p53 triggers a rapid senescence program in human tumor cells lacking functional p53. Proc Natl Acad Sci USA. 1997. 94:9648-9653.

Wang W, Yang X, López de Silanes I, Carling D, Gorospe M. Increased AMP:ATP ratio and AMP-activated protein kinase activity during cellular senescence linked to reduced HuR function. J Biol Chem. 2003. 278:27016-27023.

Wang Z, Ma L, Su M, Zhou Y, Mao K, Li C, et al. Baicalin induces cellular senescence in human colon cancer cells via upregulation of DEPP and the activation of Ras/Raf/MEK/ERK signaling. Cell Death Dis. 2018. 9:217.

Watanabe K, Reddy BS, Weisburger JH, Kritchevsky D. Effect of dietary alfalfa, pectin, and wheat bran on azoxymethane- or methylnitrosourea-induced colon carcinogenesis in F344 rats. J Natl Cancer Inst. 1979. 63:141-145.

Watson AJ, Collins PD. Colon cancer: a civilization disorder. Dig Dis. 2011. 29:222-228.

Xie Q, Wu Q, Horbinski CM, Flavahan WA, Yang K, Zhou W, et al. Mitochondrial control by DRP1 in brain tumor initiating

cells. Nat Neurosci. 2015. 18:501-510.

Yao CH, Wang R, Wang Y, Kung CP, Weber JD, Patti GJ. Mitochondrial fusion supports increased oxidative phosphorylation during cell proliferation. eLife. 2019. 8:e41351.

Yoon YS, Yoon DS, Lim IK, Yoon SH, Chung HY, Rojo M, et al. Formation of elongated giant mitochondria in DFO-induced cellular senescence: involvement of enhanced fusion process through modulation of Fis1. J Cell Physiol. 2006. 209:468-480.

Youle RJ, Karbowski M. Mitochondrial fission in apoptosis. Nat Rev Mol Cell Biol. 2005. 6:657-663.

Yu T, Robotham JL, Yoon Y. Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. Proc Natl Acad Sci USA. 2006. 103:2653-2658.

Zamorano-León JJ, Olivier C, de Las Heras N, Mateos-Cáceres PJ, Brime Menéndez R, Rodríguez-Sierra P, et al. Vardenafil improves penile erection in type 2 diabetes mellitus patients with erectile dysfunction: role of tropomyosin. J Sex Med. 2013. 10:3110-3120.

Zhan L, Cao H, Wang G, Lyu Y, Sun X, An J, et al. Drp1-mediated mitochondrial fission promotes cell proliferation through crosstalk of p53 and NF-κB pathways in hepatocellular carcinoma. Oncotarget. 2016. 7:65001-65011.

Zhang J, Block ER, Patel JM. Down-regulation of mitochondrial cytochrome c oxidase in senescent porcine pulmonary artery endothelial cells. Mech Ageing Dev. 2002. 123:1363-1374.

Zhao J, Zhang J, Yu M, Xie Y, Huang Y, Wolff DW, et al. Mitochondrial dynamics regulates migration and invasion of breast cancer cells. Oncogene. 2013. 32:4814-4824.

Zhou X, Hao Q, Lu H. Mutant p53 in cancer therapy − the barrier or the path. J Mol Cell Biol. https://doi.org/10.1093/jmcb/mjy072.

Ziegler DV, Wiley CD, Velarde MC. Mitochondrial effectors of cellular senescence: beyond the free radical theory of aging. Aging Cell. 2015. 14:1-7.