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ARTICLE TYPE

Effect of Apple, Baobab, Red-Chicory, and Pear Extracts on Cellular Energy Expenditure and Morphology of a Caco-2 Cells using Transepithelial Electrical Resistance (TEER) and Scanning Electron Microscopy (SEM)

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Abstract

The present study investigated the effects of four food extracts on the Caco-2 intestinal cell line using a new transepithelial electrical resistance method (TEER) concurrent with electron microscopy (SEM). Caco-2 cells are widely used in transepithelial studies because they can be cultured to create a selective permeable interface similar to that between the intestinal lumen and the basolateral tissue. These cells absorb, secrete, and function as a barrier that limits the passive transepithelial diffusion of hydrophilic solutes from the digestive tract into the circulation. The intercellular tight junctions provide a limiting barrier to the movement of the solutes through the paracellular route. The integrity of this tissue must be maintained when studying cellular physiology. TEER provides useful information on cellular function when a tissue in chemical equilibrium is perturbed by an external compound (such as nutrient, drug, pathogenic microorganism or toxins). In order to restore this equilibrium, the cells need to expend energy that can be calculated through a mathematical TEER value. The change in energy from the equilibrium value depends on the time elapsed and the nature and concentration of the test substance.

The results show that extracts of four commercial foods (with the total phenolic content shown in mg/g gallic acid equivalents) - apples (3.39), baobab (130), red chicory (13.31), and pears (1.15) induced concentration-dependent changes in both the energy and histology (morphology) of the cells as well as the formation of mucopolysaccharide. These changes, reported graphically and mathematically, were altered in the presence of the free radical (oxidant) 2,2'-azobis (2-amidinepropane) dihydrochloride (AAPH). At the highest concentration, measured, the food extract with the highest phenolic content (baobab) completely damage the cells.

The new simple in vitro TEER assay offers a novel approach to investigate the influence of nutrients, antinutrients, food toxicants, and drugs on the physiology and morphology of the Caco-2 cells that may predict their behavior in the digestive tract.

Introduction

The intestinal Caco-2 cell line is extensively used for cellular permeability studies of food and xenobiotics (Hidalgo et al., 1989; Berger et al., 2003; Sergent et al., 2005; Boulaaba et al., 2013; Rocha et al, 2013; Elhadidy et al., 2014; Rajkovic et al., 2014; Vazquez et al., 2014). Because these cells can be grown on porous plugs, it is possible to utilize them to create an interface (by selective permeable monolayer cells) that simulates both the epithelium of the intestinal mucosa and molecular flow between the lumen and the basolateral tissue (Hidalgo et al., 1989). Caco-2 cells absorb, secrete, and function as a barrier that limits the passive transepithelial diffusion of compounds from the digestive tract into the circulation. Because this barrier is not static (Benson et al., 2013) but is affected by a specific chemical input, it can open and close the tight junction selectively. Thus, the cells are able to control the diffusion from the lumen to the basolateral tissue and *vice versa*. The capacity of such solutes to cross the lipid membrane is limited and the major route to permeate the monolayer of epithelia is quite specific. The intercellular tight

junctions operate as a limiting barrier to the movement of the solutes through this specific path (Hubatsch et al., 2007).

Because the barrier can modulate adsorption of food-containing microbial toxins and pathogenic bacteria in the tissue and into the circulation, delaying or increasing the adsorption can determine the success of a therapy, it is important to understand the biological mechanism of this barrier in medical treatment (Sköld et al., 2006; Yaheya et al., 2009). The same considerations apply for the bioavailability of nutrients in food (Genser, 2008; McCabe-Sellers et al., 2003; Won et al., 2012).

An important characteristic of Caco-2 cell line is that the compounds added in the apical area (lumen) have to cross the membrane perpendicularly. Epithelial cells are functionally and structurally "*polarized"* and their basal-lateral extremity differs from the apical one. These properties make this *in vitro* model very suitable in studies of both morphological and biochemical responses of transepithelial and paracellular transport (Zweibaum et al., 1991; Fonti et al., 1994) under different chemical stimuli (Garaguso et al., 2013; Garaguso et al., 2011; Durazzo et al., 2010; Nobili et al., 2006).

A further property of these cells is that they are structurally similar to human adult colon epithelia (Pinto et al., 1983). Another morphological and structural aspect is that they are also nonhomogeneous; indeed this cell line comprises at least 4 cytotypes (Ferruzza etal., 2002). Caco-2 cells are neoplastic but if they proliferate on plastic discs or on polycarbonate filters, then they act like normal enterocytes, always remaining as a highly polarized (monolayer) without forming clusters typical of neoplasia. Caco-2 cells represent a good model for the study of the damage caused by free radicals and by the protective action of antioxidants (Hidalgo et al., 19891).

The principal approach used in the present study to investigate the integrity of tissues grown on a porous support is based on the transepithelial electrical resistance (TEER). This is a non-invasive technique which measures the impedance between the lumen and basolateral tissue. TEER measurements use a constant direct current (DC) applied by two electrodes, one connected with the lumen side and the other one with the basolateral side. By applying Ohm's law it is possible to measure the related cells resistance (R). If the chemical equilibrium of the tissue is perturbed by an external substance, then the cells tend to restore the equilibrium. For this purpose the cells expend energy by activating a metabolic pathway in response to the chemical injury. This is reflected in the cultured medium and in the resistance value, which is monitored by TEER.

The aim here it is to use TEER values to calculate the energy released by the cell to restore its chemical equilibrium. This energy depends on the time elapsed and chemical characteristics and concentration of the tested compound. TEER measurements provide further valuable information, not only when analyzing the conveyance of the compound through lumen and basolateral tissue or when detecting the maximum active dose of a single compound, but also about the cell physiology. The Caco-2 cell line is therefore tested in this study using hydrophilic extracts from different commercial foods such as apples Kalinowska et al., 2014), the African fruit baobab (Lamien-Meda et al., 2008; Mulaudzi et al., 2011), red chicory (D'Evoli et al., 2013), and pears (Li et al., 2014). Mathematical analysis of the data, reinforced by scanning electron microscopy (SEM), were used to determine the effect of the food extracts on the energy and histology of the cells.

Materials and Methods

Samples extraction. Commercial fresh samples of apple, baobab gel, red chicory, and pear extracts were used for creating biological triggers for the cell culture. These products were chosen because of their wide-ranging concentration of polyphenols that can interfere with cellular metabolism, especially when they are linked with carbohydrates. Each fresh sample (10 g) was extracted with a water/methanol (50:50 v/v) solution and dried by Rotavapor (Büchi, Flawil, Switzerland) at 50°C and then re-suspended in water. Next, the total free polyphenols were determined by the Folin-Ciocalteu method (Coseteng and Lee 1987). The values obtained range (in mg/g gallic acid equivalents) as follows: pears; 1.15; apples; 3.39; red chicory; 13.31; and baobab, 130. The concentrations of the tested polyphenols were: 10, 50, and 100 μg/ml for apple; 50, 500, 1000, 2500, and 5000 μg/ml for baobab; 5, 10, 15, 20, 50, 100, and 200 μg/ml for red chicory, and 10, 50, and 100 μg/ml for pear. These concentrations, with and without the pro-oxidant AAPH, were added to a monolayer of the Caco-2 cell culture. Fresh oxidation agent (AAPH) solution (6 mg/ml) was used for each sample. TEER values were then measured every 30 min along with a control sample without the extracts or AAPH.

TEER Analysis. TEER values were tested on Caco2 cells in order to measure changes in the tight junction permeability (Lamien-Meda 2008) using a Millicell-ERS (Millipore Corporation, Billerica, MA, USA) at a constant voltage of 0.1 mV. The cells were seeded onto a polycarbonate filter cell culture chamber inserts with the following dimensions: diameter, 6.5 mm; area, 0.33 cm^2; pore diameter, 0.4 μm (Costar Corporation Cambridge MA UK), at a density of 1.5×10^5 cells per filter and placed into a Falcon multiwell plate (Costar Transwell; Corning Incorporated, NY, USA). The filter separated the chamber into two parts (apical and basal) which represent the lumen and the basal area of the gastroenteric system, respectively. The TEER was then measured in these two chambers to assess the tight-junction permeability and cell damage. These parameters were adopted to detect the early intestinal barrier function *ex vivo* damages. Each measurement was performed three times and the results are reported as means and standard deviations.

The human colon adenocarcinoma cell line (Caco-2) was provided by A. Zweibaum (INSERM, Villejuif, Paris, France). The cells were cultivated '*in vitro'* in Falcon™ plastic bottles with a surface of 75cm^2 (Becton, Dickinson and Co., Milan, Italy). The culture medium consisted of Dulbecco (DMEM) containing 25 mM of glucose, and 3.7g/l $NaHCO_3$ further supplemented with 4 mM of L-glutamine, 10% of fetal horse serum, 1% non-essential amino acids, 10^5 U/l penicillin and 100 μg/l of streptomycin. The cells were incubated at 37°C at a 90% humidity in an atmosphere consisting of 95% air and 5% CO_2. The culture medium was replaced three times a week until the attainment of the cell confluence.

Each tray cell represented an independent bowel microculture. In this way, with only a few multiwell trays, we were able to obtain a considerable number of cultures, which were monitored daily. Ideal conditions for confluence and differentiation were noted after 14-18 days..

Two fundamental parameters were evaluated: TEER between the apical and basal-lateral compartments (Szczesny, 1978) and the observation of the morphology of the increasing epithelia culture. The latter was observed with the Diavert inverted microscope (Leitz GmbH, Germany).

SEM Analysis. The cellular monolayer was fixed for 12 hours in formaldehyde (10%), gluteraldehyde (2.5%) and then dried with alcohol solution. Next, the sample was dehydrated by Critical Point Drying (Emitech K850, Quorum Technologies, West Sussex, UK). The final dehydration was achieved in CO_2. Finally, the sample was sputtered with gold for 120 sec at 30 mA in a

modified atmosphere with 2% Argon and analyzed using SEM (EVO LS10, Carl Zeiss Microscopy GmbH, Jena, Germany).

SEM was used to determine the formation of mucopolysaccharide by the Caco-2 cells induced by added food extracts and the pro-oxidant. The Goblet cells, one of the cell types comprising the Caco-2 cell line, are known to produce mucopolysaccharide (Dorofeyev et al., 2013; Alvarenga et al., 2014). When the Goblet cells produce mucopolysaccharide, they are positioned between the microvilli forming a matrix well visible by SEM, but not by optical microscopy**.**

Mathematical treatment and interpretation of the data. The introduction of a chemical compound induces a modification of the cellular chemical equilibrium. In order to restore the equilibrium, the cells expend a certain amount of energy. The energetic variation might be interpreted in terms of the cellular reaction to the chemical interference. In order to estimate the energy variation of the cells, we developed a simple mathematical model based on the Joule effect, which relates the power dissipated by the circuit with the resistance [in this case TEER, R(t)] and the current flowing through the cells:

$$P(t) = R(t) \times I(t)^2 \quad (1),$$

where P(t) is the calculated power in watts, R(t)) is the resistance, the time-dependent measured TEER value (in Ohms), and I the current(in Amperes), flowing through the circuit that is kept at a constant voltage of 0.1 mV. The time variation of the TEER is due to the modification of the chemical composition of the cellular medium, which is caused by the added compounds, the consequent chemical response of the cells, and the electric current. The numerical integration of Eq. (1) over the time results in:

$$E = \int_{in}^{fin} P(t)dt \quad (2)$$

This equation provides an estimate of the energy released (in Joules) by the cell due to the passage of an electric current. We calculated the energy (ΔE) and power (ΔP) differences between the non-equilibrium situation in the cell in the presence of an added food extract by using equation 3:

$$\Delta E = E_{food\ extract\ or\ free\ radical} - E_{control};\ P = P_{food\ extract\ or\ free\ radical} - P_{control} \quad (3)$$

Two different energy situation can occur depending on the sign of ΔE:

1) $\Delta E < 0$: the energy amount spent by the control is larger than that of the sample with antioxidant or pro-oxidant. The cells still try to regain the chemical equilibrium.

2) $\Delta E > 0$: the energy amount spent by the control is lower than that of the sample with antioxidant or pro-oxidant. In this case, the cells cannot regain equilibrium. If the interference persists, however, then severe damage to the cells occurs, as further verified by SEM analysis of the cell morphology.

The outputs, plotted as a function of the different antioxidant or prooxidant concentrations (see the Results and Discussion sections) are interpreted as the amount of cell energy that is used to regain the equilibrium.

In this study only the R (Resistence) and the V (Volt) have been determinate by the instrument. The energy parameters have been calculated with equations 1-3.

Results

The aim of this work is to present a new approach to identify a threshold above which the cell cannot regain its physiological equilibrium after being exposed to an inflammatory stimulus. The results obtained represent the energy difference between the control and the sample with added oxidant (ΔE) determined by TEER as function of time. ΔE starts from zero then becomes negative because the cells expend energy to achieve the chemical equilibrium. By increasing the oxidant concentration, the energy difference could reach a positive value. This can occur when the oxidant concentration is so high that it is incompatible with a complete cellular recovery of equilibrium. We interpret the region of positive value as a threshold above which the ΔE relative to the concentration of tested compound could severely affect the cellular equilibrium. Indeed, we identified a concentration threshold above which the cells cannot recover their equilibrium. These observations are supported by SEM data of control and treated cells..

Figure 1 shows the result with the apple, baobab, red chicory and pear extracts. In the apple samples without added free radical oxidant (AAPH), all the TEER values (Fig. 1B) are less than the control value. By contrast, with added oxidant, all TEER values in Figure 1 are greater than the control. The same figure also shows the TEER values of baobab extract. The TEER values without oxidant are greater than the control value, except for the 5000 μg/ml concentration. At all tested concentrations, the TEER values with oxidant (AAPH) added are greater than the control value. In red chicory, the TEER values are always less than those of the control. This trend is more pronounced in the presence of the oxidant (AAPH). At the end of this figure, we show the TEER pear values in which the values are quite close to the control value except for the 100 μg/ml concentration, which illustrates a negative trend. We repeated the experiment using the free radical or oxidant (AAPH). The TEER values are similar but with a more pronounced trend.

Figure 2 illustrates the energy differences values for apple, baobab, red chicory and pear extracts. In the apple extract, the ΔE plot shows all the values to be in the positive field. By contrast, with added oxidant (AAPH) all energy is negative. It seems that the presence of the oxidant helps the cell to recover its equilibrium. Thus, in the baobab extract, the sample without oxidant shows a negative trend, except for the 5000 μg/ml concentration. In the samples treated with oxidant (AAPH), the energy differences are also all negative even at the threshold concentration near 5000 μg/ml.

The plots of ΔE support the TEER results (Figure 1). The energy expenditure is greater and reaches a maximum value of 333 nanojoules (nJ), which is much larger than the maximum value (12.6 nJ) for the samples without added oxidant. It seems that the presence of oxidant induces an additional effect, which may be due to the action of free radicals in the culture medium.

Regarding the observed results with pear extract, it appears that the changes in energy shown in this Figure 2 reflect the TEER behaviour (Figure 1), in which all the ΔE values are negative except for 100 μg/ml concentrations. In this trial, this third concentration (100 μg/ml) appears to be the threshold for the system. Above this concentration, it is difficult for the cell to recover the physiological equilibrium. The related ΔE value with oxidant (AAPH) reflects the energy expenditure trend: negative for the first (10 μg/ml) and second (50 μg/ml) concentrations and positive for the third one (100 μg/ml).

Figure 3 and 4 illustrate the results of the SEM analyses. The photos Control Mag 802X and control Mag 12.04 KX represent the control at the indicated magnifications.

To confirm the apple TEER results (Figure 1), we used SEM to observe changes in the cell morphology. The SEM analysis shows that after introduction of the apple extract, the cell monolayer is in physiological equilibrium; the monolayer reacts to the addition of extract with an increased secretion of mucopolysaccharide located at the level of microvilli. This reaction causes the formation of mucous cords along the edge of the apical wall of the cells (Fig. 3 C). The model with the induced peroxidative trigger shows an even greater secretion of mucopolysaccharide (Fig. 3 F). Cellular morphology, however, is not altered in both models and cell structure is perfectly preserved and functioning.

The SEM analysis of baobab samples shows the differences between the concentration of 2500 µg/ml (near the threshold) with and without oxidant. Samples with oxidant (Fig. 3 F1) show an increase of mucopolysaccharide secretion compared with those without oxidant (Fig 3 C1).

The added baobab fruit extract (5000 µg/ml) with a very high phenolic content induced major cellular damage. At the end of the experimental phase, the monolayer is completely destroyed (Fig. 3 C). The SEM results for the model with the induced peroxidative trigger (AAPH) show an increased secretion of mucopolysaccharides and even some necrotic areas. Overall, however, at this concentration the epithelium culture is preserved intact and is not changed morphologically (Fig. 3 F).

In the red chicory samples, the presence of oxidant (AAPH) induces an additional effect, which can be explained by cellular changes in the culture medium induced by the added free radicals associated with the oxidant. SEM photomicrographs (Fig. 4 C) show that the cell monolayer is in physiological equilibrium. The red chicory extract also induces an increase in the mucopolysaccharide secretion. The flow of mucopolysaccharides, located at the apex of microvilli, results in the formation of mucosa clumps all along the apical wall of the cells (Fig. 4 C). With the peroxidative trigger (AAPH), cell damage is apparent with deep necrotic areas. This extract seems to have a low antioxidant activity in counteracting the peroxidative effect (Fig. 4 F).

The SEM results show that a moderate level of mucopolysaccharide secretion with a normal morphology seems to occur at the concentration of 50 µg/ml (near the threshold) with (Fig. 4 F1) and without oxidant (Fig. 4 C1). The SEM analysis seems to show that the cellular monolayer is in physiological equilibrium. After the introduction of the pear extract (100 µg/ml), the cells react with an increased mucopolysaccharide secretion. Such a secretion, located at the microvilli level, forms a dense glycocalyx that makes it difficult to observe the apical cells (Fig. 4 C). In the model with the trigger-induced peroxidation, a more pronounced type of secretory mucopolysaccharide accumulation is observed (Fig. 4 F). In both cases, however, the cellular morphology is not modified. The apical structure of the cells is preserved and functional.

Conclusions

Our results show that it is possible to measure the cellular reaction to different chemical stimuli by means of simple electrical measurements. Adding a chemical compound or a food produces an inflammatory stimulus. Consequently, the cell tries to regain the physiological equilibrium. The cells use different mechanisms to inactivate the added prooxidant or food extract that generate chemical stimuli. For instance, cells can overcome adverse effects of added compounds through use of detoxifying enzymes, including intestinal cytochromes P450 (CYPs) (Thummel and Wilkinson, 1998; Lin and Lu, 2001; Paine et al., 2006; Gibbs and Hosea, 2003), esterase (Liederer and Borchardt, 2006;), uridine diphosphate glucuronosyl transferase (UGTs) (Peterson et al., 2005; Chang et al., 2007), and sulfonotrasferases (SULTs) (Coughtrie and Johnston, 2001; Nishimuta et al., 2007; Nishimuta et al., 2005).

As observed in the present study, another cellular defense is to delay the effect of external substances through secretion of mucopolysaccharides. The mucopolysaccharides provide a protective barrier. Yet a third possibility is to avoid adverse effects that involve penetration into the cell by using a permeability barrier with the P-glycolprotein (Huang et al., 2010) and organic anion transport polypeptides (Won et al., 2012; Bailey et al., 2007). The mechanisms of defense influence the composition of the cultural medium and resistance to the electrical current because these need energy to be activated and are strictly related to the concentration and nature chemical compounds in the medium.

The calculated energy expenditure (ΔE) can be positive or negative, compared to a control that is set equal to zero. When (ΔE) is negative, the TEER behaviour is always greater than the control, and the cells are not affected by the presence of the tested compound. If the energy expenditure is positive, then the TEER value is always lower than the control and the cells cannot regain their physiological equilibrium. The histological and morphological analysis support these suggestions: the cells are always alive and the morphological differences are related to chemical modifications which can be measured by the observed energy expenditure.

In this study, the tested extracts containing variable amounts of polyphenols. If these are present as glycosylates, then they can penetrate into the cellular membrane and interfere with the enterical oxidative cytochrome P-450 enzymes (CYP-450). If the polyphenols concentration is too high, then the cellular detoxification is more difficult and a degenerative process takes place, as strikingly illustrated above with the data for red chicory. In our trials we applied extreme stress conditions, because the tested compound remains on the cell surface for a long time. This situation is not usual in a normal gastrointestinal environment, in which there is a regular flow of compounds so that the contact with the cell surface is reduced. We, therefore, believe that this study provides a simple model to detect the maximum compound concentration that can be tolerated by the Caco-2 cell line without irreversible cellular damage. This model could be useful for further studies of cell physiology, using other single compounds at different concentrations or a combinations of nutrients, microbial and plant toxins (Friedman and Rasooly, 2013) or drugs under physiological conditions.

The findings of the present study merit extension to demonstrate the general value of this new bioassay to nutrition and other biomedical sciences. Finally, it would also be of interest to find out whether the described method using Caco-2 cells can replace a more complex method based on monkey kidney Vero cells we used to measure the toxicological activities of native and inactivated toxins, including ricin produced by castor beans (Rasooly et all., 2012), Shiga toxin (Stx2) produced by *E. coli* bacteria (Rasooly et al., 2014), and *Staphylococcus* enterotoxin A (SEA) produced by *Staphylococcus aureus* bacteria (Friedman et al., 2011).

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Figure 1 Results of TEER Apple, Baobab, Red Chicory and Pear

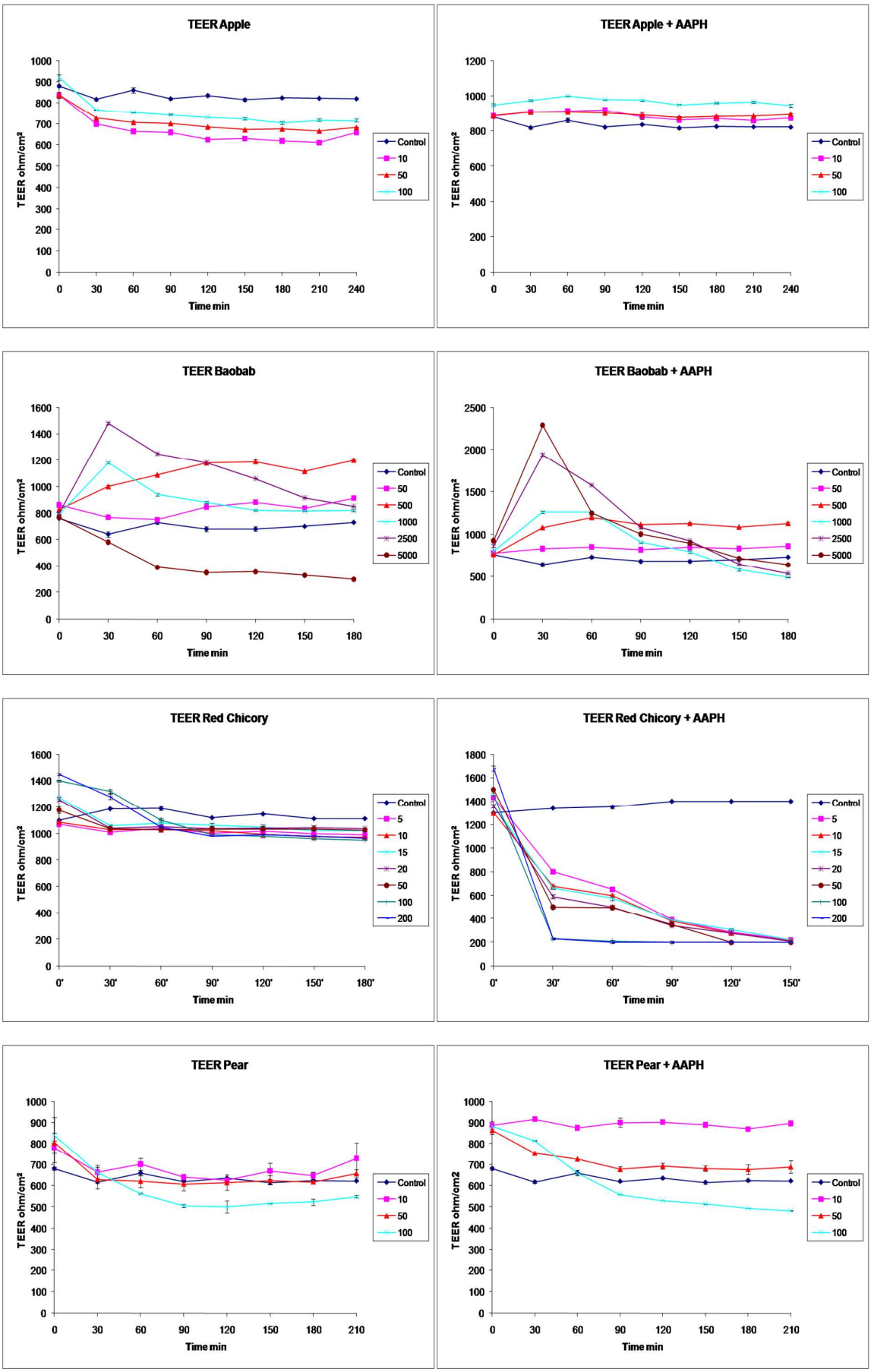


Figure 2 Results of Energy difference regarding Apple, Baobab, Red Chicory and Pear

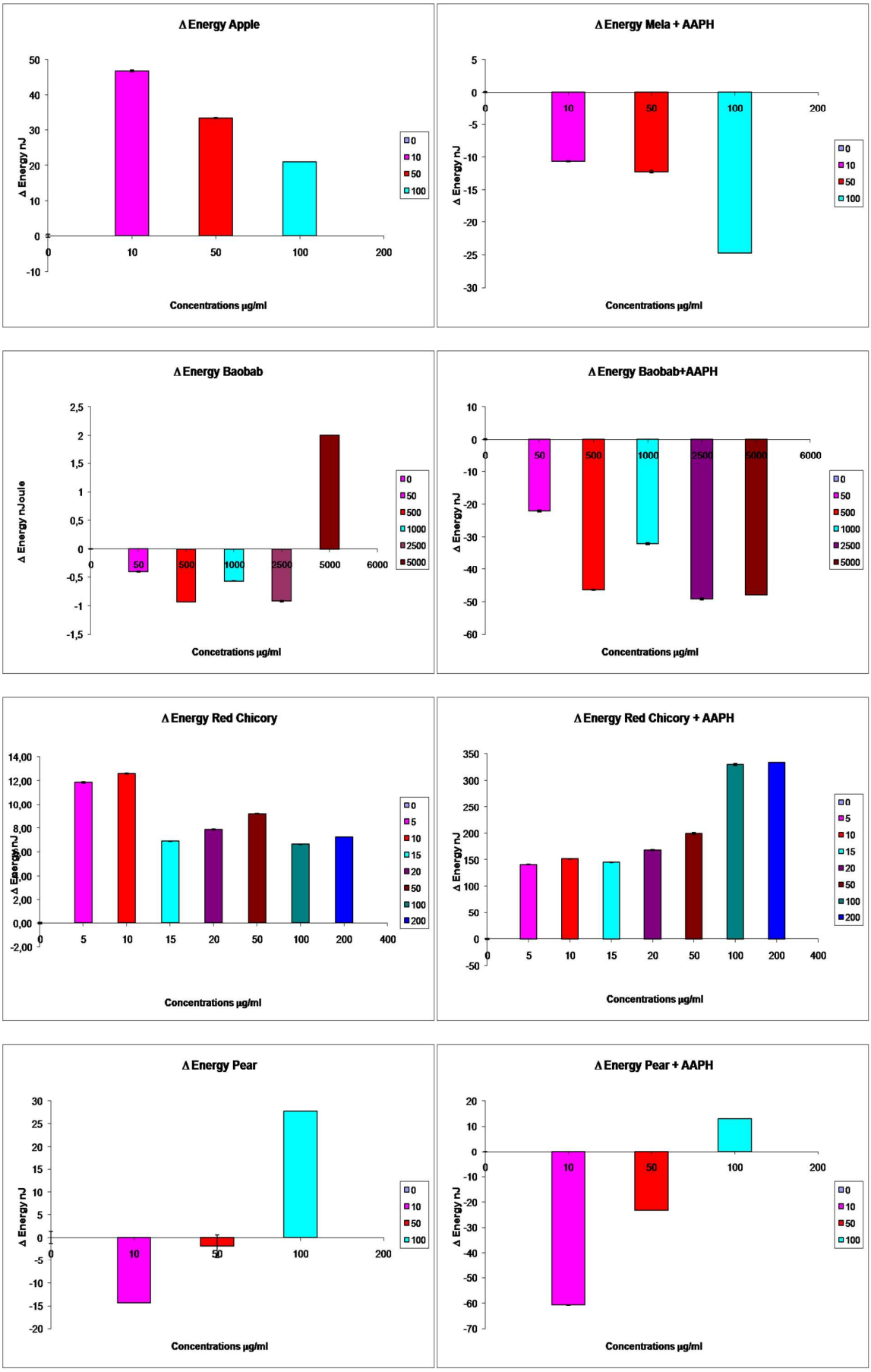
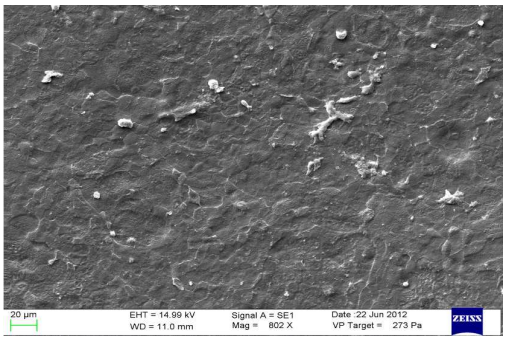
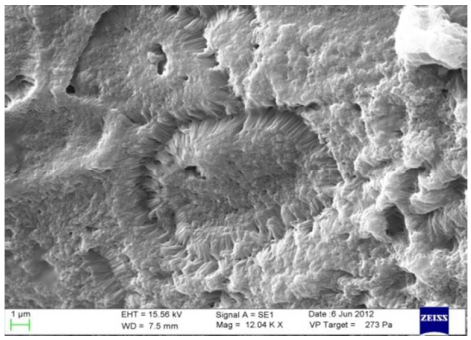


Figure 3 Results of SEM analysis regarding Control, Apple and Baobab



Control Mag 802X



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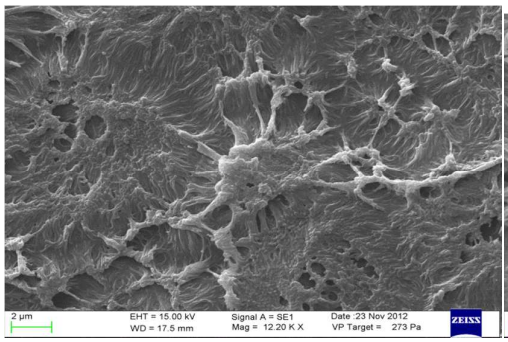
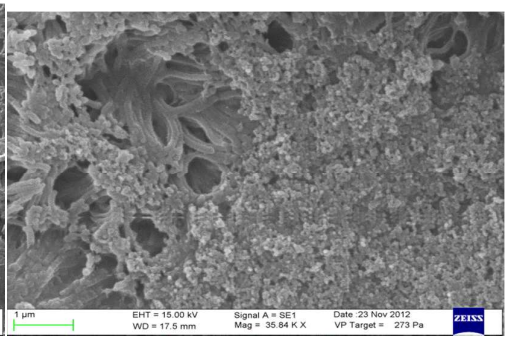
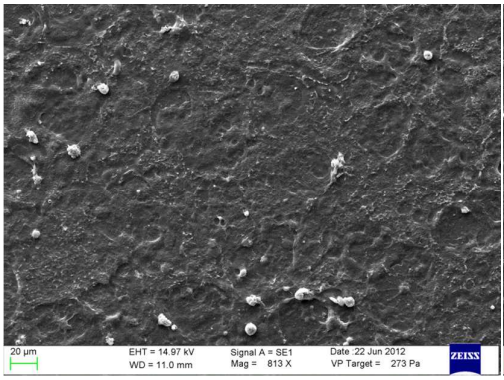


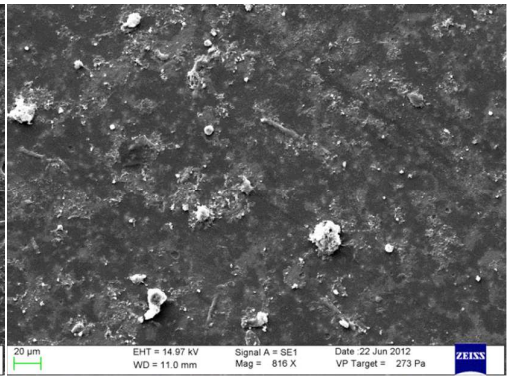
Apple (C)



Apple (F)



Baobab (C1)



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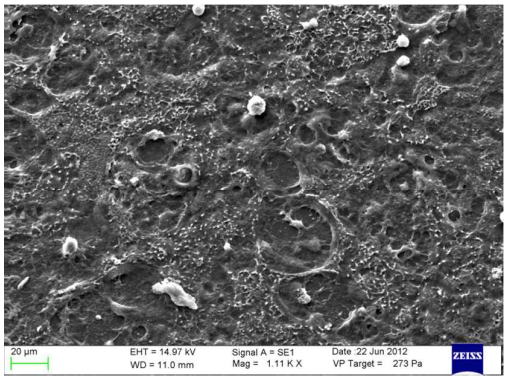

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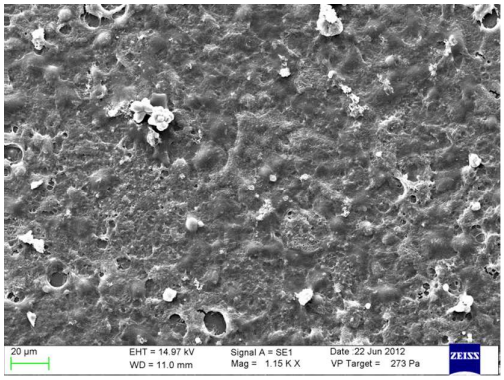
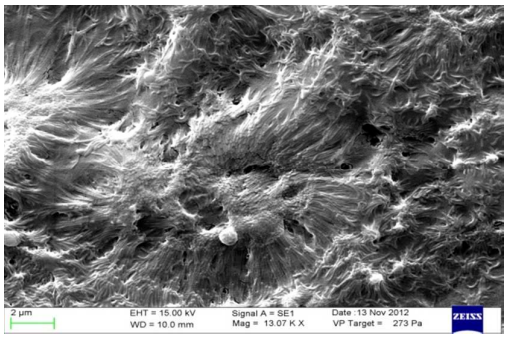


Baobab (F)

Figure 4 Results of SEM analysis regarding Red chicory and Pear



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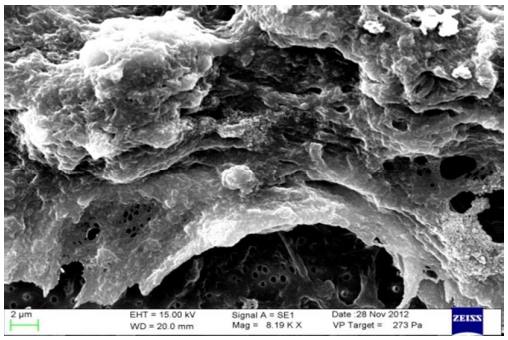

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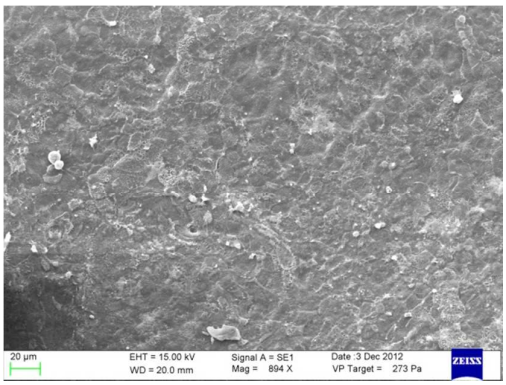
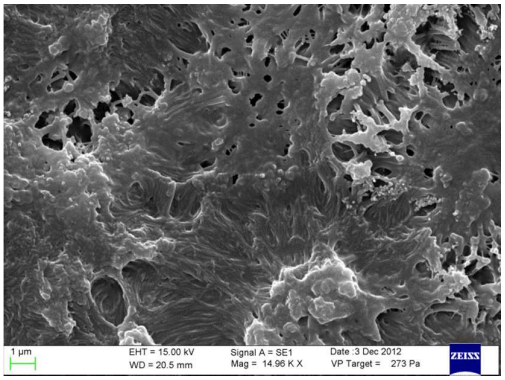
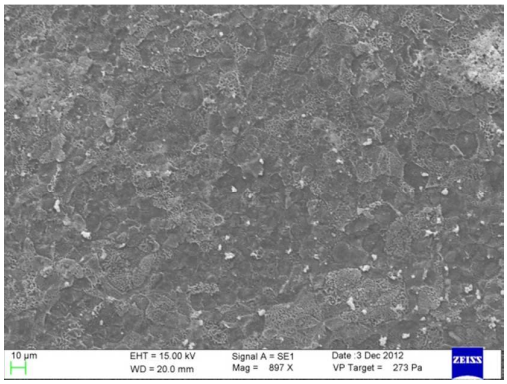


Pear (C1)



Pear(C)



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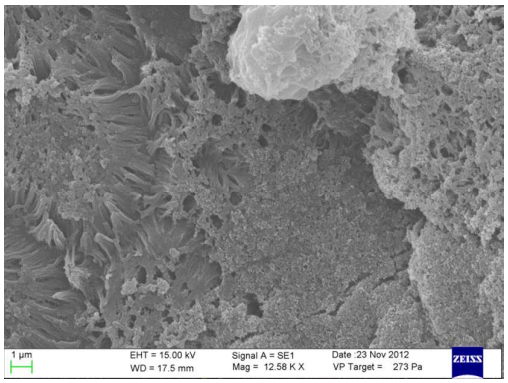

Pear(F)