Learning is an active process emphasizing purposeful interaction and the use of knowledge in a meaningful environment. Scientific experiments are, by nature, inquiry-based activities; developing scientists must learn to propose hypotheses, design experiments, and select appropriate materials. Many cognitive psychologists have portrayed learning as a process of creating individual meaning and understanding from personal experiences, a perspective referred to as constructivism (Ormond, 1998). Greater retention via long-term memory storage and future retrieval is thought to be a significant advantage of constructivism. In this model, students are encouraged to assume responsibility for their own learning (Figure 1). Students gain understanding by participating in activities in a laboratory setting and, thus, teachers should think about how to provide opportunities for active mental processing by students (Gabler & Schroeder, 2003). For example, diverse environments encourage inquiry and build new knowledge, allowing students to recognize the need for change. Moreover, encouraging students to apply knowledge while practicing skills promotes higher-level thinking and fosters a greater understanding of major concepts. The successful application of constructivism to student learning requires a few key factors:

1. prior knowledge in the content area in the form of lectures, directed readings, and group discussions
2. attainment of conceptual meaning (e.g., apoptosis and cancer)
3. articulation of experimental design and application (i.e., pre-laboratory proposal)
4. sufficiently structured experiences (i.e., laboratory exercise).

A laboratory exercise is presented that incorporates constructivist principles into a learning experience designed for upper-level university biology courses. There are typically 15-20 students in our laboratory and students work in groups of three to four. Two full laboratory sessions are required for the completion of this experiment. The specific objectives for this exercise are as follows:

1. to introduce students to cancer biology and to the regulation of programmed cell death as part of the cell cycle
2. to engage students in scientific inquiry through experimental design and testing, using a constructivist approach
3. to encourage cooperative learning in a scientific laboratory setting
4. to promote scientific thinking and writing in the form of a laboratory report.

While we have designed this experience primarily for college courses, it may also be adaptable to certain high school curricula. The exercise is aligned with several of the National Science Education Standards (NRC, 1996). This laboratory experience is aligned with the Science as Inquiry standard in that students are exposed to the basic elements of the scientific method within a constructivist framework. The students are required to formulate a hypothesis and to adapt and execute the experimental design to address the hypothesis. Students are supported in this process by instructor-facilitated review of the pre-laboratory group proposal in order to enhance the experimental design while maintaining maximum student input. Additionally, the post-laboratory discussions guide students to reflect on the experimental design and to analyze and interpret the data as well as to consider the process of scientific inquiry. In this way, the students engage the scientific method within an instructor-controlled environment. The post-laboratory assessment also encourages students to construct new hypotheses and to modify the experimental design based on the interpretation of their data consistent with the History and Nature of Science standard. Introductory lectures and group discussions facilitate an expanded understanding of the conceptual framework (i.e., cancer biology and cell cycle regulation) of the exercise as articulated in the Life Science (The Cell) content standard. The relevance of this laboratory experience to cancer therapy and prevention is aligned with the personal and community health concept outlined in Science in Personal and Social Perspectives standard.
Apoptosis

Maintenance of tissue integrity in multicellular organisms requires a precise balance between cell proliferation and programmed cell death, or apoptosis. Apoptosis is a highly-regulated process that is morphologically characterized by cell shrinkage, nuclear pyknosis, chromatin condensation, fragmentation of DNA, changes to the distribution of specific lipids within the plasma membrane, and blebbing of the plasma membrane (Danial & Korsmeyer, 2004). Apoptosis normally functions as part of a cell cycle checkpoint to prevent damaged cells from proliferating, and the deactivation of this apoptotic pathway strongly correlates with oncogenic transformation. Various naturally occurring polyphenolic compounds have a cytotoxic effect on tumor cells by “reactivating” apoptosis. For example, we have shown that polyphenolic-rich extracts, isolated from cranberry fruit, can induce apoptosis in breast (MCF-7) and colon (HCT-116 and HT-29) cancer cell lines (Griffin et al., 2005). Using a fluorescent TUNEL assay to access the extent of apoptosis within cell populations, the polyphenolic-rich extracts were shown to induce apoptosis in the oncogenic cell lines at a very high frequency (90%) while the matched normal cells remained relatively unaffected (<15%) (Griffin et al., 2005). These results suggest that polyphenolic compounds may hold some chemopreventative or chemotherapeutic promise. This observation serves as the basis of this exercise that uses a modified TUNEL assay to investigate apoptosis in human cheek cells.

Experimental Design

Although numerous assays exist for the detection of apoptosis within a cell, those relying on the detection of DNA fragmentation, such as the TUNEL assay, have become the gold standard. The most significant occurrence during apoptosis is the fragmentation of genomic DNA as this event irreversibly commits the cell to death. DNA fragments can be examined in a variety of ways. For example, the presence of DNA fragments as a “ladder” on an ethidium-stained agarose gel has long been used as a diagnostic tool for apoptosis. However, this assay has limited sensitivity and specificity, and requires lengthy preparation. These limitations have led to the development of newer methods that take advantage of the free 3'-hydroxyl group at the ends of the DNA fragments. For example, the TUNEL assay relies on an enzyme, terminal deoxynucleotidyl transferase (TdT), to incorporate a specific label onto the free 3'-hydroxyl groups of DNA fragments; the labeled DNA fragments can then be visualized with either a fluorescent or histological detection method. The TUNEL assay substantially reduces preparation time, allows for apoptosis to be detected in vivo, and provides more quantitative information.

The utility and relative simplicity of the TUNEL assay makes it useful for an inquiry-based laboratory. For example, investigation into the effect(s) of extracellular signals on apoptosis can be easily accomplished with this assay. We propose here a laboratory exercise, using the TUNEL assay, which allows students to investigate the effects of over-the-counter (OTC) products that contain polyphenolic compounds

### Table 1. Over-the-counter products with bioactive polyphenolic compounds. Some suggested products for use as treatment variable with the TUNEL assay.

(Personal observation; Murphy et al., 2003; Bhat & Pezzuto, 2001; Seeram et al., 2005; Gupta, Hussain & Mukhtar, 2002).

<table>
<thead>
<tr>
<th>Product</th>
<th>Active polyphenolic(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranberry juice</td>
<td>Proanthocyanidins (PACs)</td>
</tr>
<tr>
<td>Grape juice</td>
<td>Triterpenes (TTPs)</td>
</tr>
<tr>
<td>Pomegranate juice</td>
<td>Ursolic acid (UA)</td>
</tr>
<tr>
<td>Green tea</td>
<td>Resveratrol</td>
</tr>
<tr>
<td>Red wine</td>
<td>Ellagitannins</td>
</tr>
<tr>
<td>Urinary Health Tablets (CVS)</td>
<td>Anthocyanins</td>
</tr>
<tr>
<td>CranSupport (Natrol)</td>
<td>Epigallocatechin-3-gallate</td>
</tr>
<tr>
<td>Fitness Cranberry (Life Fitness)</td>
<td>Resveratrol</td>
</tr>
<tr>
<td>Bounty Cranberry (Nature’s Bounty)</td>
<td>PACs, TTPs, UA</td>
</tr>
<tr>
<td>AZO Cranberry Supplement</td>
<td>PACs, TTPs, UA</td>
</tr>
</tbody>
</table>

### Table 2. Pre-lab form for treatment variable selection.

<table>
<thead>
<tr>
<th>Product</th>
<th>Concentration</th>
<th>Treatment Time</th>
</tr>
</thead>
</table>

(Table 1) on apoptosis in their own epithelial cells. Consistent with a constructivist approach, each group selects a different product, and determines the appropriate product concentrations and treatment length.

Figure 2. Outline of experimental design and approach.

Pre-Laboratory: Group Proposal of Experimental Design

Each group of students should submit a proposal prior to the initial laboratory session that includes a student-derived hypothesis and an outline of the proposed experimental design (Table 2). Comparisons of treatment variables can be part of both a pre- and post-laboratory discussion (Figure 2).

### Method

The following materials are required for the exercise described:

- FragEL DNA Fragmentation Detection Kit (EMD Biosciences, QIA33 for colorimetric detection or QIA39 for fluorescent detection)
- glass microscope slides/coverslips
- wooden toothpicks (flat)
- 0.1% poly-L-lysine
- glass capillary tubes (or eyedropper)
- microcentrifuge tubes

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Laboratory Session #1

This portion of the protocol is identical for both labeling methods. A clean glass slide was covered with a thin film of 1% poly-L-lysine solution to facilitate cell adhesion. Cheek cells were obtained by gently scraping the inside of the student's cheek with a flat toothpick. The cells were then spread onto the portion of each glass slide covered with the 1% poly-L-lysine solution, and allowed to adhere for two to three minutes. The slide was then rinsed by immersing it in 1X Tris-buffered saline (TBS), pH = 7.4, for five minutes. The TBS was gently poured off the slide and the edge of the slide was blotted on a paper towel. The slide was then placed in a Petri dish or separate plastic container prior to the application of the treatment using the predetermined concentration(s) and incubation time(s). After treatment, the slides were washed for five minutes in 1X TBS, and then placed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH = 7.4. At this point, slides may either be stored in the fixative until the next laboratory session, or removed from the fixative after a 30 minute (room temperature) incubation and stored in PBS until the next laboratory session. If the cells are stored in the fixative, a wash (10 minutes at room temperature) should be done prior to labeling to remove excess fixative.

Laboratory Session #2

For the histological labeling, slides containing fixed cheek cells were washed in 1X TBS for five minutes at room temperature. The cells were then covered with 100 μl of proteinase K solution (20 μl/ml), and incubated at room temperature for exactly 20 minutes. The slide was rinsed with 1X TBS briefly. The cells were then covered with 100 μl of 3% H2O2 (diluted in methanol) and incubated at room temperature for exactly 10 minutes. Following a brief rinse with 1X TBS, the cells were covered with diluted 1X TdT equilibrium buffer and incubated at room temperature for 20 minutes. The equilibrium buffer solution was carefully blotted from the slide by placing the corner of an absorbent towel at the edge of the slide. The cells were then immediately covered with 60 μl of TdT labeling reaction mixture, covered with a glass coverslip, and incubated at 37°C for 1.5 hours. After incubation, the coverslip was removed and the cells rinsed with 1X TBS. The cells were then covered with 100 μl of stop solution and incubated at room temperature for five minutes. The cells were again rinsed with 1X TBS and then covered with 100 μl blocking buffer and incubated for 10 minutes at room temperature. The cells were then immediately covered with 100 μl diluted 1X conjugate, and incubated at room temperature for 30 minutes in a humidified chamber. After incubation, the cells were rinsed with 1X TBS and covered with 100 μl DAB solution for 10 minutes. The cells were then rinsed with dH2O, and, if desired, immediately covered with 100 μl methyl green counterstain solution and incubated for 20 minutes. The cells were then rinsed with two changes of 100% ethanol, and one change of xylene substitute (Clear-Rite, Electron Microscopy Sciences).

For the fluorescent labeling, slides containing fixed cheek cells were washed in 1X TBS for five minutes at room temperature. The cells were then covered with 100 μl of proteinase K solution (20 μl/ml), and incubated at room temperature for exactly five minutes. The slide was rinsed with 1X TBS and the cells were then covered with 100 μl of 1X TdT equilibrium buffer and incubated at room temperature for 20 minutes. The equilibrium buffer solution was carefully blotted from the slide by placing the corner of an absorbent towel at the edge of the slide, and the cells were immediately covered with 60 μl of TdT labeling reaction mixture, covered with a glass coverslip, and incubated at 37°C for 1.5 hours. After incubation, the coverslip was removed and the cells were rinsed in three changes of 1X TBS for one minute each.

For both labeling methods, mounting media was placed on the cells followed by a glass coverslip, and the edges were sealed with nail polish. Slides may be examined immediately, or stored for several weeks in a dry, dark location. A representative set of images of labeled cheek cells is shown in Figure 3.

For the histological labeling, examination of the slides can be done with any standard compound microscope. Histological labeling is visible as either a brown (apoptotic nuclei) or green (normal nuclei) color (Figure 3D). Non-apoptotic cells should be predominantly spherical while apoptotic cells should be irregularly-shaped ovoids and pyknotic (Figure 3D). For the fluorescent labeling, an epifluorescent microscope with standard DAPI (UV) and FITC filter sets is required. The total cell population may be visualized by DAPI labeling, and nuclei should all appear blue (Figure 3C). Apoptotic nuclei can then be visualized by FITC labeling, and true apoptotic nuclei will appear a vibrant green color (Figure 3B). For both labeling methods, apoptosis rates were determined based on a simple percentage of total cells that were labeled. Care should be taken to count all cells within a field of view and as many cells should be counted as possible.

Results & Discussion

The TUNEL assay has numerous educational advantages. For example, the students are able to investigate the effects of a
A given compound on apoptosis rates in their own cheek cells. Students could incubate the cheek cells briefly using the OTC products (Table 1), and determine if apoptosis rates are increased in comparison to untreated cheek cells. Since the results can be obtained by simply counting the number of labeled and unlabeled cells, sophisticated quantitative analysis need not be employed, and either fluorescent or histological labeling may be used. Fluorescent labeling requires a relatively expensive epifluorescence microscope which may make histological labeling a more practical approach for some. We have found that the apoptosis frequency calculated using both methods is very consistent, but the extent of DNA damage in individual nuclei cannot be quantitated using the histological labeling method. Thus, the TUNEL assay exercise described here provides students the opportunity to design and execute their own experiment, and exposes them to the implementation of the scientific method through an inquiry-based investigation.

Assessment

The learning experience is assessed at both a group and individual level. Classwide comparisons of the data may greatly enhance learning, and provide the instructor with a useful group assessment tool. Directed questions provided by the instructor that encourage students to reflect on their laboratory experience and evaluate their experimental design can facilitate discussion. For example, class discussion can be stimulated with simple questions such as: 1) Was a correlation observed between the active polyphenolic(s) in the OTC product selected and the rate of apoptosis in the cheek cells? Or, 2) How did concentration and treatment length affect apoptosis rates? Also, encouraging students to make connections between knowledge of the cell cycle and cancer research (i.e., Why care about apoptosis?) can further enhance the learning experience.

In our classroom, each student is required to submit a written laboratory report to allow for individual assessment by the instructor. Consistent with the constructivist approach, individual responses to effective follow-up questions, such as those addressed in the classwide discussions, can be included in the “Discussion” section of the written report. This allows the instructor an opportunity to assess the ability of students to make connections beyond the scope of the experiment.

Conclusion

Knowledge is enhanced when students are actively engaged in the learning process. When this is coupled with guidance and scaffolding from the instructor, students are able to gain a better understanding of complex concepts (Ormond, 1988). Experimental design and execution provide students with an opportunity to create and explore science, staying true to a constructivist approach to learning. In these laboratory exercises, students learn how to design an experiment, test a hypothesis, and discuss results with classmates while learning about cell cycle regulation and basic cancer biology. Students are better able to reappraise new knowledge to the topic from participation in this exercise, and in this way “construct” their own learning.

References


