THE EFFECTS OF FOLIC ACID ON CELLULAR SENESCENCE
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ABSTRACT
Biological functions of folic acid (FA) have been reported in various cells to include synthesis and repair of DNA. We have shown that over-dosage of FA causes blood haemolysis, leading to progressive anaemia. It is reported that 200–400 μg/ml of FA increases the size of blood cells within 2h of treatment leading to abnormal cell division and necrosis. This observation suggests early cellular aging or senescence. We hypothesized that FA may play a vital role in cellular senescence. Kidney fibroblast (cos7) cells were treated with 200 μg of FA in combination with an anti-neoplastic agent, nocodazole prior to FA treatment. FA affected the expression of fibronectin (protein marker for aging) after nocodazole exposure. Furthermore, fibronectin expression was higher in FA-treated cells of the 14th generation compared to the 8th generation. 14th generation cells also showed a decrease in cell size when exposed to FA treatment. Contrasting previous results, treating cancer cells with FA showed in fact cell-beneficial effects as FA over-dosage has a noticeable effect on cell aging. However, these effects may be cell type or tissue-specific.

INTRODUCTION
The significance of folic acid (FA) in biological functions like biosynthesis of nucleotides and re-methylation of homocysteine, and synthesis of DNA and its repair has been reported in many cell types. Studies have shown that folic acid acts as a co-factor, and helps in cell division and growth (Wikipedia). Folate deficiency is correlated to serious health problems including neural tube defects in developing embryos (Wolff & others, 2009). Therefore women consume FA during early stages of pregnancy. FA also helps in the production of red and white blood cells, and its deficiency may lead to anemia, leading to fatigue, weakness and inability to concentrate (Zalusky & Herbert, 1961).

In the field of aging FA is a largely debated topic. While many articles and health websites suggest FA supplements to decrease the rate of aging, several researchers believe FA plays detrimental roles in the health of certain patients. Canada, the USA, and many other nations currently fortify foods such as wheat and cereals with FA. It is also a readily available over-the-counter drug. Therefore the uncontrolled/unaccounted consumption of FA is increasing day by day.

We previously observed that FA over dosage causes blood haemolysis, a positive sign for progressive anaemia. 200–400μg/ml of FA caused an abnormal blood cell division and necrosis suggesting early cellular aging or senescence. Cells derived from embryonic...
tissues can only divide to a finite number of times known as the Hayflick limit (Hayflick & Moorhead, 1961). After amount of cell divisions growth arrests and the cells may or may not die (reviewed in Hayflick, 1985; Hayflick, 1994). As cells approach this limit in cell divisions they accumulate fibrous proteins like fibronectin (Kumazaki et al., 1991; Dumont et al., 2000).

I hypothesize that FA plays a vital role in cellular senescence. In order to investigate this viability, morphology and senescence of kidney fibroblast cells (cos-7) will be compared to cells exposed to an anti-neoplastic agent, FA and a combination of the two. Similar experiments will be conducted with cos-7 cells from two different generations, P8 and P14.

RESULTS
FA rescues cells from nocodazole-induced cell death process and drives cellular senescence: FA treatment caused only minimal changes in the cell viability of the naturally growing Cos-7 cells (This could be due to specific experimental conditions or due to the cell type itself.), although when induced with nocodazole, it seemed to rescue the cells from the damaging effects of the anti-neoplastic agent and increased cell viability (Fig. 1). Dramatic changes in cell sizes occurred with FA treatment. In both the naturally growing and nocodazole-treated cos-7 cells Addition of nocodazole and FA, it seemed to rescue the cells from the damaging effects of the anti-neoplastic agent and increased cell viability (Fig. 1). Dramatic changes in cell sizes occurred with FA treatment. In both the naturally growing and nocodazole-treated cos-7 cells. Combined these results possibly represents a phase in the cell growth where cells undergo proliferation, but are also steered towards aging (Fig. 3)

Older generations show a larger increase in FA induced expression of fibronectin: Compared to naturally aging cells of generations P8 and P14, FA treatments promotes increased cell aging. FA treatment did not have any significant effects on the cell viability of the generations (Fig. 4), although it has significant effects on the cell sizes. FA reduced cell sizes in both generations with a larger effect shown on the P14 cells (Fig. 5). Similarly, FA treatment increased the fibronectin expressions in both the P8 and P14 cells a marker of cellular senes-
The effects of folic acid on cellular senescence. The trend was again greater in the P14 cells. (Fig. 6) This suggests that FA has a negative impact on both young and older generations of cells, but its impacts on older generations are far more damaging and drastic.

DISCUSSION
FA is a common dietary supplement consumed by many people around the world. Although widely debated, many nations including Canada and the US are currently fortifying foods such as wheat and cereals with FA. Due to opinions in various media sources many individuals consume FA as an additional dietary supplement in the belief that it reduces the cell aging process. Consumption of FA is increasing in unprecedented levels with limited research into negative side-effects. We have demonstrated the harmful effects of FA over-dosage (200µg-800µg) on blood cells. These current findings suggest that FA plays a large role in cell senescence process- but its effects are dependent on type of senescence (natural vs. induced) and cell type. FA should therefore be carefully administered depending on the health status of an individual.

FUTURE DIRECTIONS
FA is a drug that may have cell specific effects. Therefore I want to test the affects of FA on various cell types. It has been suggested that FA is beneficial for cancer patients due to its cell proliferative properties. A recent study illustrated FA’s affects on apoptosis of epithelial cells in the gastric cancer patients (Cao & others, 2005). FA is often recommended and prescribed in cancer-like disease cases to help support a patient’s red blood cell production. However, FA over-dose can have deleterious effects in certain cancers suggesting a dual role in cancer-protection against tumor initiation and progression of pre-neoplastic cells (Smith et al., 2008). Future studies will investigate these specific issues.

MATERIALS AND METHODS
Cells were cultured through standard protocols until enough cells were obtained to perform viable tests. Equal amounts of cell suspension
1 x 106 cells/well were used during each test. Cells were then treated with either 200 μg/ml FA in buffered saline (pH 7.2) and/or 100μg/ml nocodazole. All experiments were carried out in triplicates.

**Cell Viability**
Cells were stained with trypan blue dye and viewed under a light microscope. Blue coloured cells were considered as dead cells, while unstained cells alive.

**Cell Morphology**
Cells were additionally stained for a to visualize cell morphology under a light microscope. Pictures were captured using a camera attached to the microscope and analyzed using Image-J.

**Cell Senescence**
Protein samples were distributed on nitrocellulose paper and blocked with 5% skimmed milk resuspended in 15ml phosphate buffered saline-tween 20 (PBS-T). The blot was washed 3 times with PBS-T and treated with primary antibodies specific for fibronectin. A secondary antibody that recognizes the primary antibody was used to visually detect fibronectin via chemiluminescence. Photos were analyzed with Image-J.

![Figure 6](image)

**Figure 6** FA treatment increased the fibronectin expressions in both the P8 and P14 cells, indicating clear signs of senescence, although the P14 cells had a much more prominent and rapid effect. This again indicates that FA has a negative impact on both young and older generations of cells, but its impacts on older generations are far more damaging and drastic.

### ABBREVIATIONS
- DNA: Deoxyribonucleic acid
- UV: Ultra Violet
- FA: Folic Acid
- PBS-T: Phosphate Buffer Saline - Tween 20

### KEY WORDS
- Folic acid; Cellular senescence; Dietary fortification; Kidney fibroblast cells

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I am grateful to my mentor Dr. Ashim Bagchi for his valuable guidance, suggestions and expert knowledge that helped me work on this challenging project. I thank Dr. Pawan Singal for providing facilities in his laboratory at the Institute of Cardiovascular Sciences. I am very thankful to the Child Health Foundation of Manitoba for sponsoring my research to the Canada-Wide Science Fair 2012 held in Charlottetown, Prince Edward Island. I express my sincere gratitude to my parents for their unconditional support and encouragement.

### REFERENCES
7. Hayflick, L. How and Why We Age. Ballan-

REVIEW OF "THE EFFECTS OF FOLIC ACID ON CELLULAR SENESCENCE"
Reviewed by: Shobha Ramsubir, PhD
Senior Manager, Business Development & Research, Ontario Genomics Institute

Overview
The study aims to examine the cellular effect of over-doses of folic acid (FA) with respect to aging. COS-7 cells were treated with FA and the effects on cell viability, morphology and fibronectin expression were assessed. The author hypothesized that FA plays a role in senescence and concluded that FA promotes senescence. In the literature, senescence is defined as the irreversible loss of cell division capability for a cell. Some of the common methods used to assess senescence are telomere length assessment, DNA synthesis assays or examination of biomarkers such as Ki67/PCNA. One of the best characterized methods is the use of the β-galactosidase (β-Gal) assay as senescent cells have been shown to over-express β-Gal. The use of the latter assay would have strengthened this study. It was good that the author attempted to look at different generations of cells for this study.

The article contains numerous grammatical, spelling and punctuation errors that need to be corrected to make it publication-ready. The writing also needs to be strengthened as at times it is unclear what the author is trying to communicate eg. “A recent study illustrated FA’s affects on apoptosis of epithelial cells in the gastric cancer patients (Cao & others, 2005).” The effect demonstrated is not given and so this does not help build evidence for the point it is supporting. Some sentences throughout should be re-written to be more direct.

Abstract
The hypothesis is clearly stated but the abstract does not frame the rationale for the study. The rest of the paper relates the importance of the study to the fact that FA is widely prescribed with unknown effects but this is not introduced here. 200-400 ug/ml of FA was mentioned but it was unclear if these were in vitro or in vivo levels. It is mentioned that the function of FA varies by cell type but no indication is given as
to the choice of COS-7 as model cell line and what the normal effects are in this cell type. The abstract should summarize the results concisely but are more general statements that an effect was observed. It should be re-positioned to say what this effect was (increase or decrease in a particular factor examined).

Introduction
This section better frames the study. The reference to Wikipedia should be replaced with a reference from the primary literature and other data introduced are not referenced at all. This would be a good opportunity to expand on the pleiotropic effects that are alluded to in various sections but not really clarified.

Results
In general, all bar charts should have error bars and some basic statistical analysis performed that report the number of replicates. The discussion of the figures remains general and do not describe the data in depth as one would expect in this section. Figures with multiple images should be labelled as “(a), “(b)” etc and each image discussed.
Fig 1-3. What generation of cells were used for this assay?
Fig 2. “Dramatic changes in cell size” is reported in response to addition of FA. In Fig 1 it suggests that FA rescues cells from the effect of nocodazole but this is not reflected in Fig 3.
Fig 3. The dot-blot assay is not really discussed in the results section or the figure title.
Fig 4. Title refers to cell size data but none is shown in this figure.
Fig 5. The figure mentions a more “rapid” effect on P14 cells but time data is not shown or discussed. It is not clear what arrows in the H&E staining are indicating.
Fig 6. It is not clear what the arrow is used to indicate other than re-iterating increased expression.

Discussion
This section is concise and the author attempts to draw conclusions from the results and relate it to a real-world application. He states “We have demonstrated the harmful effects of FA over-dosage (200µg-800µg) on blood cells.” This is likely reference to a prior study that is not referenced. If it is not published data, then it should be indicated as such.

Future Directions
The author states: “It has been suggested that FA is beneficial for cancer patients due to its cell proliferative properties.” After submission of this article, a new study has suggested otherwise in breast cancer. During revision, the author should look at this study as he plans future experiments – Manshandi et al. PLOS One. 2014

Materials and Methods
Although mentioned in the body of the study, this section should mention the cell type used. The methods of the dot-blot assay are not described. The method of staining for cell morphology assessment should be described. Image-J software supplied should be referenced. Antibodies used should be listed as this section should allow someone to replicate the study if desired.

Reviewer recommendation
Due to the need for major revisions to both the body of the article and the figures, I do not recommend this article be published in its current state. The author’s curiosity and interest in this type of work is commendable. Also commendable are the number of different techniques he attempted in this project.