

# A COMPARATIVE ASSESSMENT OF FOLIC ACID-INDUCED CELLULAR SENESCENCE

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The biological functions of folic acid (FA) including the prevention of neural tube defects in developing embryos and synthesis of DNA and its repair, have been well reported in literature. As a result; the fortification of folic acid into many daily foods in Canada such as wheat and cereals is mandatory. 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, also leading to abnormal cell division and necrosis (in vitro concentrations). This observation suggests early cell senescence. We hypothesized that FA may play a vital role in cellular senescence. 5th generation Kidney fibroblast (COS-7) cells were treated with 200 µg of FA in combination with an anti-neoplastic agent, nocodazole- prior to FA treatment. FA increased the expression of fibronectin (protein marker for aging) of nocodazole treated cells. Furthermore, fibronectin expression was higher in FA-treated cells of the 14th generation compared to the 8th generation. 14th generation cells also showed a larger decrease in cell size when exposed to FA. This suggests FA over-dosage has an affect on the cell growth cycle.

## 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 (Wagner, 1995). 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 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 (Choumenkovitch et al., 2002).

We previously observed that FA over-dosage causes blood haemolysis, a positive sign for progressive anemia. 200–400µg/ml of FA caused an abnormal blood cell division and necrosis suggesting early cell senescence (unpublished data).

Cellular Senescence is a state of permanent cell-growth arrest resulting from transitions and accumulations of deleterious cell aging processes including stress and damage. Cells derived from embryonic tissues can only divide to a finite number of times known as the Hayflick limit (Hayflick & Moorhead, 1961). After a certain amount of cell division the cells adopt a state of senescence where cell growth is limited due to a cell growth arrest program, leading to an irreversible loss of cell division (reviewed in Hayflick, 1985; Hayflick, 1994). As cells approach this limit in cell division they accumulate fibrous proteins like fibronectin (Kumazaki et al., 1991; Dumont et al., 2000).

I hypothesize that FA influences cellular senescence in COS-7 cells. In order to investigate this, viability, morphology and senescence (Hayflick 1961, 1985, 1994) were examined. Fibronectin levels of COS-7 cells with and without supplementation of the anti-neoplastic stress agent; Nocodazole were assessed by Dot-blot Assay using anti-fibronectin antibody (Vera-Cabrera L & Others 1999). The anti-neoplastic agent inhibits cell division and hence further ages the COS-7 cells. The cells were exposed to FA, Nocodazole and/or a combination of the two treatments. Similar experiments were conducted with COS-7 cells from two different generations, P8 and P14.

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The significance of folic acid (FA) in biological functions like biosynthesis of nucleotides and re-methylation of

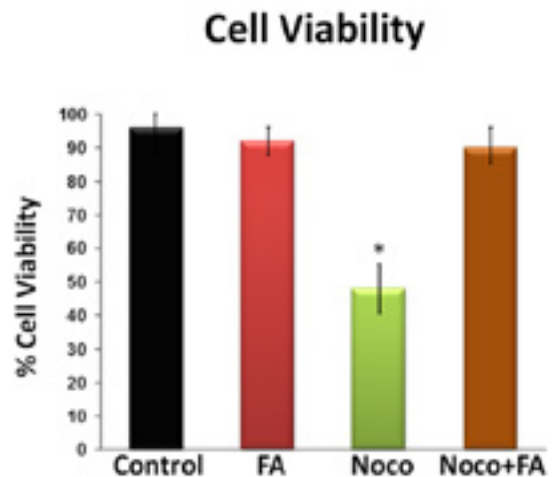
homocysteine, and synthesis of DNA and its repair has been reported in many cell types. Studies have shown that folic acid acts as a co-factor, and helps in cell division and growth (Wagner, 1995). Folate deficiency is correlated to serious health problems including neural tube defects in developing 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).

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#### FA RESCUES CELLS FROM NOCODAZOLE-INDUCED CELL DEATH PROCESS:

**Figure 1.**

Viability was assessed using Trypan Blue assay. Data was recorded, mean  $\pm$  SD values was calculated and t-test was applied ( $P < 0.05$ ):

FA treatment individually caused only minimal changes in the cell viability compared to untreated 5th generation COS-7 cells. The nocodazole treated cells showed dramatic decrease in cell viability; which is understandable since the anti-neoplastic agent is supposed to inhibit cell division and hence further age the cells. Addition of FA, to the cells pretreated with nocodazole seemed to rescue the cells from the damaging effects of the anti-neoplastic agent and increased cell viability

cell division and hence further ages the COS-7 cells. The cells were exposed to FA, Nocodazole and/or a combination of the two treatments. Similar experiments were conducted with COS-7 cells from two different generations, P8 and P14.

#### RESULTS:

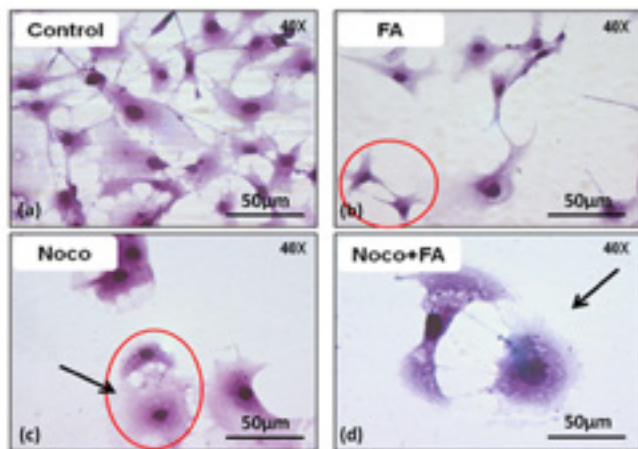
*FA rescues cells from nocodazole-induced cell death process and drives cellular senescence:*

FA treatment caused only minimal changes in the cell viability compared to untreated 5th generation COS-7 cells. Addition of FA, to the cells pretreated with nocodazole 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-pre-treated cells, FA treatment caused large increase in cell sizes. A more prominent occurring of this effect was observed in nocodazole-pre-treated cells (Fig 2). Folic acid supplementation increased the fibronectin expression in nocodazole pre-treated COS-7 cells. Combined these results possibly represent a phase in the cell growth process where are steered towards senescence (Fig. 3).

*Older generations show a larger increase in FA induced expression of fibronectin:*

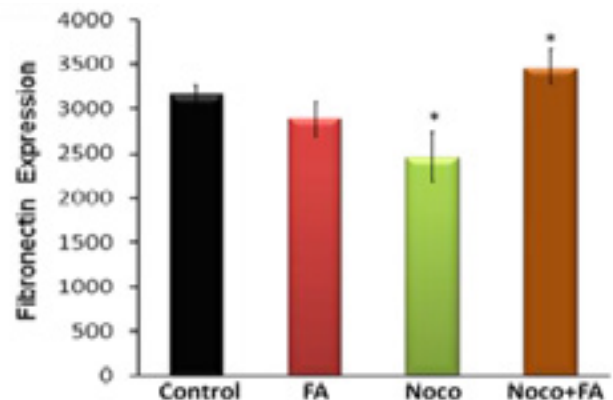
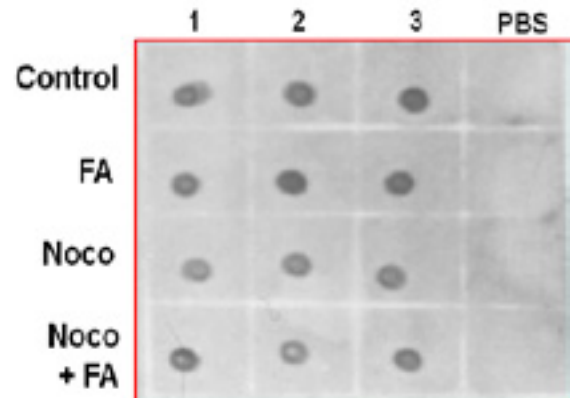
When compared to naturally aging cells of generations P8 and P14, FA treatment promotes increased cell



### IN NOCODAZOLE PRE-TREATED COS-7 CELLS, FOLIC ACID EXPOSURE LEADS TO ABNORMAL INCREASE IN CELL SIZE:

**Figure 2.**

- 2 hours after treatment, pictures were captured using a camera attached to the microscope and analyzed using Image-J software.
- FA treatment individually did not cause any prominent changes in cell sizes.
- The nocodazole pre-treated cells showed swelling present in the cells.
- The exposure of FA to the sizes even further. It is speculated that this was caused due to protein synthesis within the cells. Nocodazole pre-treated cells enlarged the cell

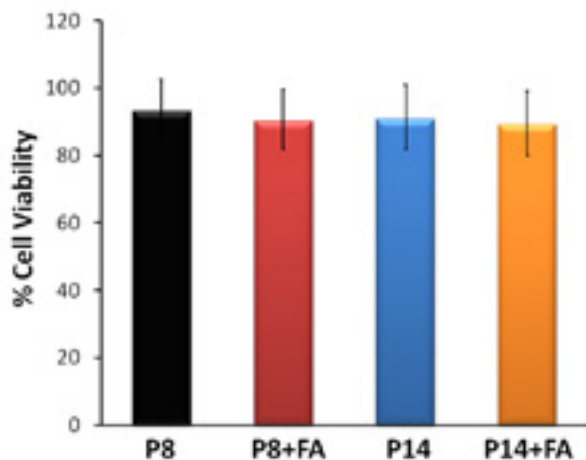


### IN NOCODAZOLE PRE-TREATED COS-7 CELLS, FOLIC ACID DRIVES CELLULAR SENESCENCE:

**Figure 3.**

Senescence was investigated by Dot-blot Assay using anti-fibronectin antibody. Data was recorded, mean  $\pm$  SD values was calculated and t-test was applied ( $P < 0.05$ ):

- Developed x-ray film of protein blots, displaying anti-fibronectin antibodies: Fibronectin expression of each blot is dependant on blot intensity which was analyzed with Image-J software.
- FA individually did not have any significant impact upon the fibronectin expression of the cells, but nocodazole decreased expressions. This impact most likely occurred due to the reduced cell numbers caused by the anti-neoplastic agent. Folic acid supplementation increased the fibronectin expression in nocodazole pre-treated P5 generation COS-7 cells. This appears to represent a phase in the cell growth process where cells undergo proliferation, but are also steered towards aging



**FA TREATMENT DID NOT HAVE ANY SIGNIFICANT EFFECTS ON THE CELL VIABILITY OF THE GENERATIONS:**

**Figure 4.**

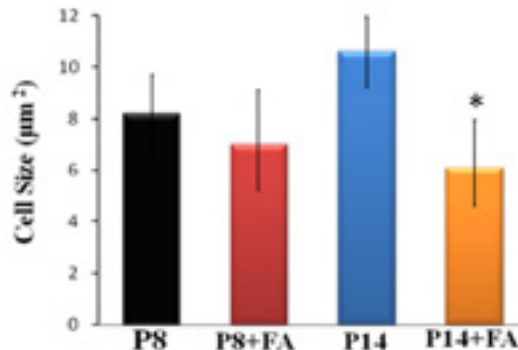
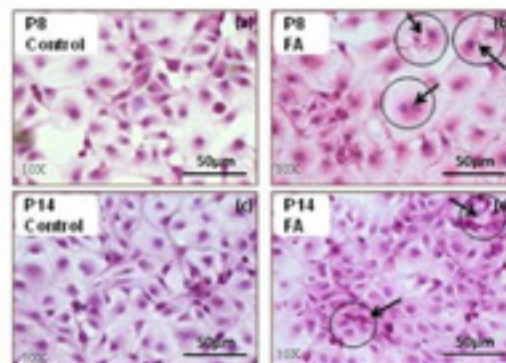
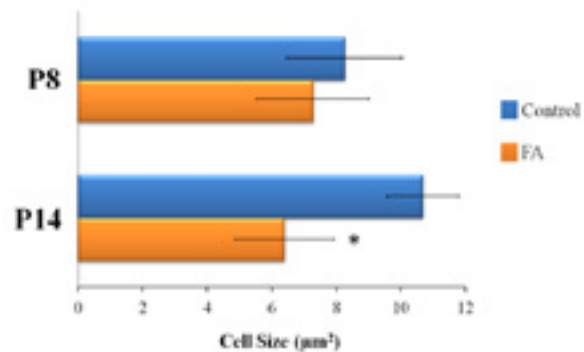
Viability was assessed using Trypan Blue assay. Data was recorded, mean  $\pm$  SD values was calculated and t-test was applied ( $P < 0.05$ ):

No prominent differences in cell viability was observed in either of the cell generations; possibly due to experimental conditions.

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, but with a larger effect shown on the P14 generation of cells (Fig. 5). Similarly, FA treatment increased the fibronectin expressions in both the P8 and P14 generation of COS-7 cells, but 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

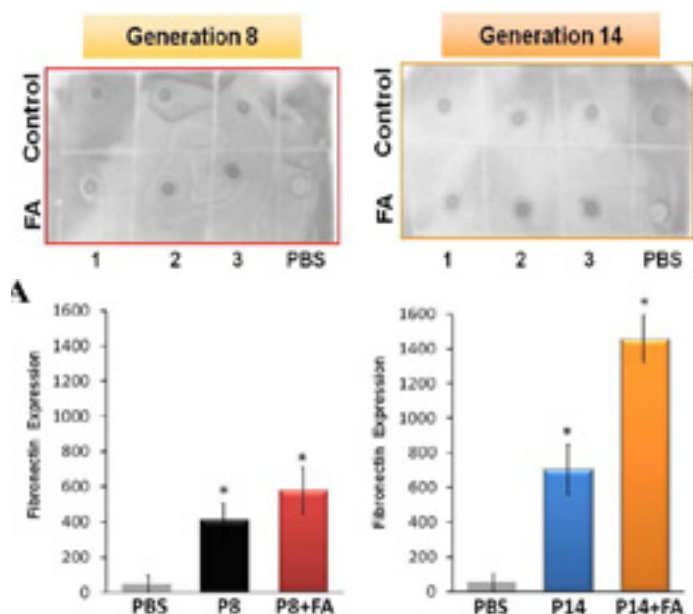


**FA REDUCED CELL SIZES IN BOTH GENERATIONS, BUT WITH A LARGER EFFECT SHOWN ON THE P14 GENERATION:**

**Figure 5.**

2 hours after treatment, pictures were captured using a camera attached to the microscope and analyzed using Image-J software. Data was recorded, mean  $\pm$  SD values was calculated and t-test was applied ( $P < 0.05$ ):

A slight decrease in cell sizes was observed in the FA treated P8 cells, but was noted as insignificant. In the FA treated P14 cells a much more significant decrease in cell size was noticed, suggesting that FA has a more prominent impact on the older cell generations.



**FA TREATMENT INCREASED THE FIBRONECTIN EXPRESSIONS IN BOTH THE P8 AND P14 GENERATION, BUT THE TREND WAS GREATER IN THE P14 CELLS :**

**Figure 6.**

Senescence was investigated by Dot-blot Assay using anti-fibronectin antibody. Data was recorded, mean  $\pm$  SD values was calculated and t-test was applied ( $P < 0.05$ ):

- Developed x-ray film of protein blots, displaying anti-fibronectin antibodies: Fibronectin expression of each blot is dependant on blot intensity which was analyzed with Image-J software.
- FA treatment increased the fibronectin expressions in both the P8 and P14 generation of Cos-7 cells, but the trend was again greater in the P14 cells, suggesting 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.

in unprecedented levels with limited research into negative side-effects (Choumenkovitch et al., 2002). We have previously demonstrated the harmful effects of FA over-dosage (200 $\mu$ g-800 $\mu$ g) on blood cells which include blood haemolysis, abnormal cell division and necrosis. 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). 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. A study has illustrated that FA plays an important role towards the chemoprevention of gastric carcinogenesis by enhancing apoptosis of epithelial cells in the gastric cancer patients (Cao et al., 2005). A 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 (Ebbing M et al., 2009); suggesting a dual role in cancer-protection against tumor initiation and progression of pre-neoplastic cells (Smith et al., 2008; Young-In Kim, 2008). Future studies will investigate these specific issues.

## MATERIALS AND METHODS:

COS-7 cells (Kidney Fibroblast Cell line; African Green Monkey) were cultured through standard protocols until enough cells were obtained to perform viable tests. Equal amounts of cell suspension, 1 x 10<sup>6</sup> cells/well, were used during each test. Then, in order to study FA's impacts on stressed and naturally aging COS-7 cells, cells were either pre-treated (2h) with 100 $\mu$ g/ml nocodazole with/without the presence of 200  $\mu$ g/ml FA in buffered saline (pH 7.2), and/or treated with 200  $\mu$ g/ml FA in buffered saline individually. All experiments were carried out in triplicates.

### Cell Proliferation:

The number of cells was calculated by a haemocytometer. For each dosage, 10 $\mu$ l of cell suspension was loaded in the counting chamber and counted using a light microscope.

### Cell Viability:

0.4g of Trypan Blue Powder was dissolved into 100ml of buffer saline to create Trypan Blue solution. Treated cells were stained with trypan blue dye (0.4%) and viewed under a light microscope. Cells with blue colour were considered as dead cells, while unstained cells as live cells. Both dead and live cells were counted using a haemocytometer, and the living cell percentage or viability was calculated.

### Cell Morphology:

Cells were additionally stained with haematoxylin & eosin dye to visualize cell morphology under a light microscope. Pictures were captured using a camera

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attached to the microscope and analyzed using Image-J software (Schneider, 2012).

#### **Cell Senescence:**

Treated cells were collected and centrifuged at 12000RPM for 10 min to isolate protein samples. Protein samples were distributed on nitro-cellulose paper (NCP) and blocked with 5% skimmed milk re-suspended in 15ml phosphate buffered saline-tween 20 (PBS-T). The blot was washed 3 times with PBS-T and treated with a primary and secondary antibody to bind with fibronectin (Anti-Mouse Fibronectin; Goat anti-Rabbit). Blot was then treated with 1ml Peroxide Solution to react to anti-bodies and 1ml Luminal Enhancer to cause chemiluminescence of anti-bodies when exposed to light. NCPs were developed on x-ray films and intensity of each blot on film was analyzed with Image-J software.

#### **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*

#### **ACKNOWLEDGEMENTS:**

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