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# DEVELOPMENT OF A NOVEL METABOLIC GLYCO-SENSING APPROACH WITH ANTI-AGING POTENTIAL

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## ABSTRACT

***Senescent cells, characterized by irreversible cell cycle arrest, are known to accumulate during aging and their accumulation in aged tissues, in turn, accelerates aging process and promotes age-related diseases. Therefore, targeting senescent cells is an attractive strategy for anti-aging or to delay features of aging. Here, we develop a glyco-sensing approach to specifically eliminate senescent cells using rat luminal ductal epithelial (LDE) breast milk cells as a model system. Senescent LDE cells, when treated with a metabolic sugar sialic acid (SA) under nutrient deprived condition, undergo ~3-fold ($p<0.001$) more apoptosis compared to LDE adult cells as demonstrated by TUNEL assay. On SA binding lectin assays, particularly with Maackia amurensis lectin I (MAL-I), senescent cells show more sialylation compared to the adult cells, possibly causing more apoptosis on these cells. This study is the first to show that SA supplementation can be protective to aging cells experiencing nutrient scarcity, while specifically killing senescent cells implying a possible role in anti-aging. Moreover, SA supplementation under nutrient deprived condition could be a strategic marker to distinguish senescent cells from adult or aging cells.***

***Keywords: Breastmilk cells, cellular senescence, senescent cells, normal aging, glycosylation sensing, sialic acid binding lectin.***

## INTRODUCTION

Cells are equipped with a variety of DNA repair mechanisms to cope with DNA damage (Hoeijmakers, 2009). However, these mechanisms sometimes can be faulty and unresolved DNA damage can impair various cellular functions promoting disease development and aging (López-Otín *et al.,* 2013). In the event of unsuccessful repair, cellular or tissue integrity can be maintained by cellular senescence (de Keizer, 2017). Senescent cells, which are permanently withdrawn from the cell cycle, but not eliminated by apoptosis, can prevail for prolonged periods of time and accumulate with age (Krishnamurthy *et al.,* 2004). It remains largely unknown how senescent cells avoid apoptosis. Although irreversible cell-cycle arrest is considered a hallmark of the senescent cells, recent studies have revealed senescent cells' increased secretion of various secretory proteins, such as inflammatory cytokines, chemokines, and growth factors - a persistent pro-inflammatory phenotype, called the senescence-associated secretory phenotype (SASP) (Watanabe *et al.,* 2017). Depending on the biological context, the SASP appears to be beneficial such as early in life or in an acute wound healing (Demaria *et al.,* 2014) or

deleterious such as promotion of tumors (Watanabe *et al.,* 2017). As senescent cells are known to accumulate during aging, it is believed that their accumulation in aged tissues accelerates aging and promotes age-related diseases and so, it is possible that the genetic clearance of senescent cells can delay features of aging (de Keizer, 2017). Indeed, recent studies show that the healthy lifespan of mice is increased by eliminating senescent cells in a transgenic mouse model (Baker *et al.,* 2011). Moreover, clearance of senescence cells has been shown to reduce several side-effects of the drugs, including bone marrow suppression and cardiac dysfunction, and even cancer recurrence (Demaria *et al.,* 2017). Therefore, targeting senescent cells is an attractive strategy for anti-aging or to delay features of aging (Baar *et al.,* 2017) and to increase quality of life against drug-associated side effects.

Epithelial cell is the major body cell type found in human milk that reflects the cell aging portfolio and glycoproteome framework (Visvader, 2009; Hassiotou and Geddes, 2012; Khan, 2013; Hassiotou *et al.* 2013a,b; López-Otín *et al.* 2013; Badr *et al.* 2014). The total human milk epithelial cells are influenced by different stages of lactation, removing milk during breast-feeding, and nutrient-scarcity of the maternal diet (Chen *et al.* 2010; Hassiotou *et al.* 2013a,b; Badr *et al.* 2014; Badr *et al.* 2015a,b; Badr *et al.* 2017). Although the mechanisms for these variations are not clearly understood, breastmilk epithelial cells that shape the cellular composition changes of the overall nutrient-deprived metabolome and other "ome" open the door for successful anti-aging senolytic therapies. Particularly, the development of senolytic compounds that might kill the senescent cells is urgently needed. Here, we developed a novel approach to specifically eliminate senescent cells using rat luminal ductal epithelial (LDE) breast milk cells as a model system.

## MATERIALS AND METHODS

### Materials

Sialic acid (N-acetyl 5-neuraminic acid, Neu5Ac, SA) was obtained from Santa Cruz Biotechnology (USA). $C_{12}$FDG was obtained from Setareh Biotech (USA). Annexin V-FITC was purchased from Molecular Probes (USA). APO-BrdU TUNEL kit and TO-PRO-3 were purchased from Life Technologies Corporation (USA). Phosphate-buffered saline (PBS, 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM disodium phosphate, 1.4 mM monopotassium phosphate, pH 7.5) was obtained from Technova (USA). WGA-FITC (Fluorescein conjugated wheat germ agglutinin specific for sialic acid [Neu5Ac] and N-acetyl D-glucosamine [GlcNAc], SNA-FITC (fluorescein conjugated *Sambucus nigra* agglutinin specific for sialic acid linked to α2,6-D-galactose [Neu5Acα2,6Gal], and MAL-I-FITC (Fluorescein conjugated *Maackia amurensis* lectin I specific for sialic acid linked to α2,3-D-galactose [Neu5Acα2,3Gal]) (Badr *et al.,* 2015a,b) was obtained from Vector Laboratories (USA). Lab-Tek 8 chamber were purchased from Thermo Fisher Scientific (USA). All other chemicals were purchased from Sigma-Aldrich in analytical grade quality.

### Collection of rat breast milk

Breastmilk samples were collected from fourteen female rats divided into two groups. The 10-12-month-old rats were regarded as the group of adult rats while 34-36-month-old rats were considered as the group of senescent rats. Breastmilk was collected from seven female lactating rats between 7 to 14 days postpartum (dePeters and Hovey, 2009) from each group in order to accumulate enough milk for isolation of epithelial cells. Prior to collection, each

female was separated from her pups for five hours to stop suckling and allow highest milk accumulation (Uejyo *et al.,* 2015). After anesthesia with isoflurane, 0.2 ml intraperitoneal oxytocin injection (2 IU/ml), milk was collected into disposable plastic test tubes using a small milking pump. The milk samples were kept at 20°C until centrifugation. The study protocol was approved by Animal Care Committee and was conducted in accordance with guidelines for the care and use of laboratory animals.

**Isolation of Breastmilk cells**

Shortly after collection, raw milk was diluted with equal volume of sterile PBS and centrifuged at 800g for 20 min at 20°C to separate the cell pellets from the milk fluid phase. The fat layer and skim milk were removed, and the freshly isolated cell pellets washed twice with warm PBS and stored at 20 °C.

**Isolation of luminal ductal epithelial (LDE) cells**

BD FACSAria sorter (BD Biosciences Inc., USA) was applied to selectively identify and isolate ≥ 95 pure populations highly LDE cells. The fluorescence-activated cell sorting (FACS) was done by an analytic gating strategy that excluded interference by dead cells and isolated live LDE cells based on $CK19^+$ protein expression (Hassiotou *et al.* 2013).

**Senescence-associated β-galactosidase assay**

Flow cytometry β-galactosidase assay was performed with minor modifications as previously described (Noppe *et al.* 2009). Briefly, adult and senescent LDE cells were incubated with $C_{12}$FDG (5-Dodecanoylaminofluorescein di-β-D-Galactopyranoside dissolved in high [K+] buffer containing 5 Fg/ml nigericin) for 1 h at 37 °C. After washing twice with warm PBS, breastmilk cells were analyzed quickly on a BD FACSAria flow cytometer. Dying cells were excluded from the analysis based on light scatter parameters. $C_{12}$FDG was measured with a green laser fluorescence (Ex-Max 405 nm/Em-Max 510 nm).

**Nutrient deprivation and sialic acid treatment**

Freshly isolated LDE cells ($1 \times 10^4$) in 2 mL of PBS buffer into BD Falcon tubes were supplemented with 10 mM sialic acid under nutrient deprived conditions as previously described (Badr *et al.,* 2015a,b). Control cells include treatment with 10 mM sialic acid in complete medium.

**Detection of apoptosis by flow cytometry**

Detection of apoptosis either by Annexin V-FITC or TUNEL assay was performed by flow cytometry on BD FACSAria sorter. The fluorescence intensity of single-cell suspension was recorded using a forward and side scatter gate to exclude debris and cell aggregates from the analysis. Fluorescence was detected at 532 nm for green laser. For each measurement 10.000 cells were used. For this purpose, cells were treated with 10 mM sialic acid in the nutrient deprived condition (PBS) for 120 min followed by PBS wash (15 min) and additional PBS incubation (0, 20, and 40 min), and then stained with either Annexin V-FITC or FITC-labeled anti-BrdU mAb (TUNEL) (10 min) (total time: 145, 165, and 185 min), and analyzed by flow cytometry on BD FACSAria sorter. Annexin V assay was performed as previously described (Cabinian *et al.* 2016). The TUNEL assay for detecting DNA fragmentation was carried out using the APO-BrdU™ TUNEL Assay Kit as previously described (Badr *et al*., 2015a,b). Negative controls with distilled water instead of terminal deoxynucleotidyl transferase (TdT) were run for each sample.

### Lectin-FITC staining and image analysis

Lectin staining with live cell imaging was performed on Lab-Tek 8 chamber. Adult and senescent LDE cells were treated with 10 mM sialic acid in the nutrient deprived condition (PBS) for 120 min and after very quick PBS wash or quick PBS wash plus additional PBS incubation (20 min), stained with lectin-FITC followed (5 µ/ml, 15 min, green fluorescence) by treatment with RNaseA (1 µg/ml, 15 min) and TO-PRO-3 (1 µM/ml, 15 min, blue fluorescence) (Badr *et al.,* 2015a,b). Fluorescence images were visualized at 63x Oil magnification and excitation were 365 nm, and 470 nm for blue and green fluorescence respectively. Images were captured on an advanced Delta Vision Elite imaging system and images were merged using softWoRx DMS from Applied Precision (Applied Precision Inc., USA).

### Statistical analysis

Data set are expressed as means ± S.E. of at least three independent experiments. ANOVA is used to determine statistical significance by analysis the ratio of two population variances. The observed differences were considered significant when $p < 0.05$.

## RESULTS

### Characterization of adult and senescent cells

Under approved protocol by the Animal Care Committee, breastmilk samples were collected from two groups of lactating rats between 7 to 14 days postpartum (Uejyo *et al.,* 2015) as follows: 10-12 months old (n=7) regarded as "adult" group and 34-36 months old (n=7) considered as the "senescent" group. After centrifuging raw milk, cell pellets were washed with phosphate buffered saline, the fluorescence-activated cell sorting (FACS) was performed by an analytic gating strategy that excluded interference by dead cells and live LDE cells (≥95%) were isolated based on $CK19^+$ protein expression (Hassiotou *et al.*, 2013a). The cellular senescene in the senescent group LDE cells was confirmed by β-galactosidase assays with $C_{12}$FDG as a substrate on a flow cytometer (Noppe *et al.* 2009). As shown in **Fig. 1**, β-galactosidase activity of the senescent group LDE cells was 91.17% ($p<0.001$) and that of the adult LDE cells was only 5.15% ($p<0.001$).

### Sialic acid supplementation under nutrient deprived condition promotes senolytic activity and apoptosis of senescent cells

We previously demonstrated that normal breast epithelial cells (MCF10A) could withstand nutrient deprivation (in phosphate buffered saline, pH 7.5 [PBS] only) without any measurable cytotoxicity up to 120 minutes (min) but cell death became evident at 180 and 240 min (Badr *et al.,* 2013, 2015a,b). However, we observed that supplementation of 10 mM N-acetylneuraminic acid or sialic acid (SA) to the nutrient deprived cells produced differential sialylation on the cell surface resulting in varying degree of apoptosis in various cell types (Badr *et al.,* 2015a,b). In this study, we undertook similar approach to analyze LDE cells from adult and senescent rats by apoptosis and lectin staining assays at different time points under nutrient deprived conditions in the presence of SA (ND+SA).

***Senolytic activity of adult and senescent cells under nutrient deprived condition in the absence (ND-SA) or presence of sialic acid (ND+SA).*** Like breast epithelial cells above, both adult and senescent LDE cells remained viable (>95%) up to 120 min under nutrient deprived condition without SA (PBS only) (data not shown). However, both cells became vulnerable to considerable cell death after 120

min in PBS. We first investigated senolytic activity under nutrient deprived condition in the presence (ND+SA) or absence of SA (ND-SA) using cell proliferation assay as previously described (Chang *et al.,* 2015). Senolytic activity of adult cells at either condition increased slightly over time (number of viable cells decreased by ~3% only) (**Fig. 2**). However, senolytic activity of senescent cells increased profoundly, particularly at ND+SA condition over time (percent of viable cells decreased: from 90.8 to 75.9 at ND-SA, **Fig. 2A** and 75.1 to 44.3 at ND+SA, **Fig. 2B**). Moreover, senolytic activity was more among senescent cells compared to the adult cells at each time point at either condition (ND-SA or ND+SA). More importantly, senolytic activity was favored under ND+SA condition compared to ND-SA (as much as 55.7%, **Fig. 2B**). In complete medium in the absence (CM-SA) or presence of SA (CM+SA), senolytic activity of both adult and senescent cells was less (7.1-16.4% in CM-SA and 19.8-30.6% in CM+SA) compared to the corresponding nutrient deprived condition, but the trend was the same as the nutrient deprived condition (**Fig. 3)**.

***Apoptosis (early and late) of adult and senescent cells under nutrient deprived condition in the absence (ND-SA) or presence of sialic acid (ND+SA).*** On annexin V-FITC assay, LDE cells from adult group under ND-SA showed 4.2-9.6% apoptosis (4.2%, 4.9%, and 9.6% at 145, 165, and 185 min, respectively) (**Fig. 4A).** In contrast, LDE cells from senescent group had almost 4-6 times higher cell apoptosis compared to the adult group at the same condition (25.6%, 29.2%, and 40.5% at 145, 165, and 185 min, respectively) (**Fig. 4A).** On TUNEL assay under ND+SA, senescent LDE cells suffered 15.7-17.3% apoptosis, while adult LDE cells had 2.6-6.4% apoptosis (**Fig. 4A).** Apoptosis of adult and senescent cells under ND+SA condition was strikingly increased. On Annexin V assay, adult and senescent cells showed 24.9-42.1% and 57-65.5%, respectively cell death in 145-185 min (**Fig. 4B**). On TUNEL assay, adult cells showed cell death 9.2-12.1% in 145-185 min; whereas cell death for the senescent cells was 29-35.1% for the same condition (**Fig. 4B**). In complete medium in the presence of SA (CM+SA), the apoptosis (based on TUNEL assay) of senescent cells was 8-12%; however, that of adult cells was negligible (**Fig. 4C**).

**Senescent cells treated with sialic acid under nutrient deprived condition (ND+SA) preferentially stained *Maackia amurensis* lectin I (MAL-I)**

As cells are known to metabolically uptake SA under nutrient deprived condition and modulate cell surface sialylation (Badr *et al.,* 2013, 2015), we examined ND+SA treated live LDE cells (adult and senescent) by lectin staining with WGA-FITC (Fluorescein conjugated wheat germ agglutinin specific for Neu5Ac [sialic acid, SA] and N-acetyl D-glucosamine [GlcNAc], SNA-FITC (Fluorescein conjugated *Sambucus nigra* agglutinin specific for Neu5Ac linked α2,6 to D-galactose [Neu5Acα2,6Gal], and MAL-I-FITC (Fluorescein conjugated *Maackia amurensis* lectin I specific for Neu5Acα2,3Gal) (Badr *et al.,* 2015a,b). While WGA and SNA showed comparable staining on both adult and senescent cells, MAL-I preferentially stained senescent cells (**Fig. 5A**). In complete medium in the presence of SA (CM+SA), lectin staining of both adult and senescent LDE cells was comparable (**Fig. 5B**).

**Figure 1**

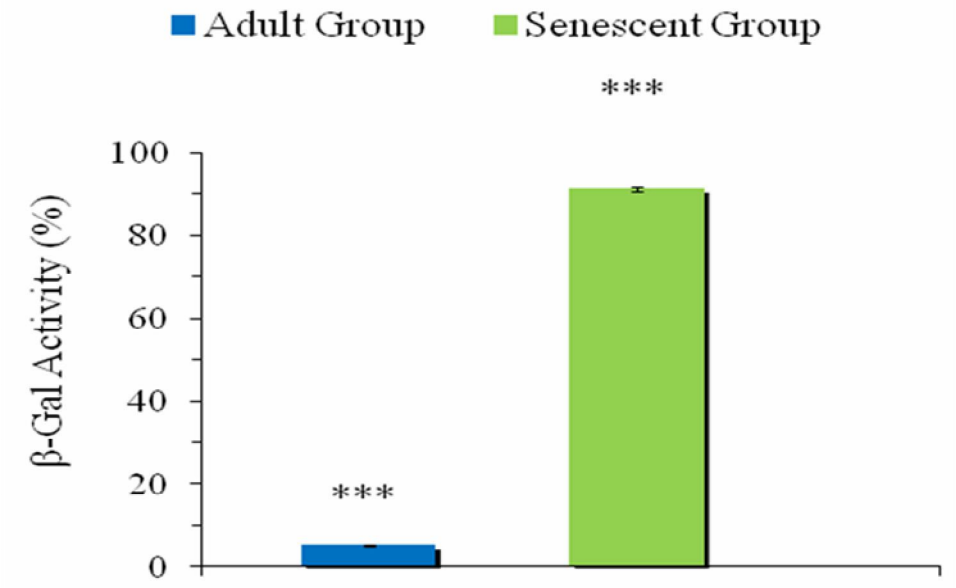


**Fig. 1** β-Galactosidase activity of adult and senescent LDE cells. Cells were incubated with $C_{12}$FDG and β-galactosidase activity was measured on a flow cytometer as described in Experimental Procedures. The data are representative of three independent experiments with S.D. indicated by error bars. *p* value <0.001 is indicated by three asterisks.

**Figure 2**

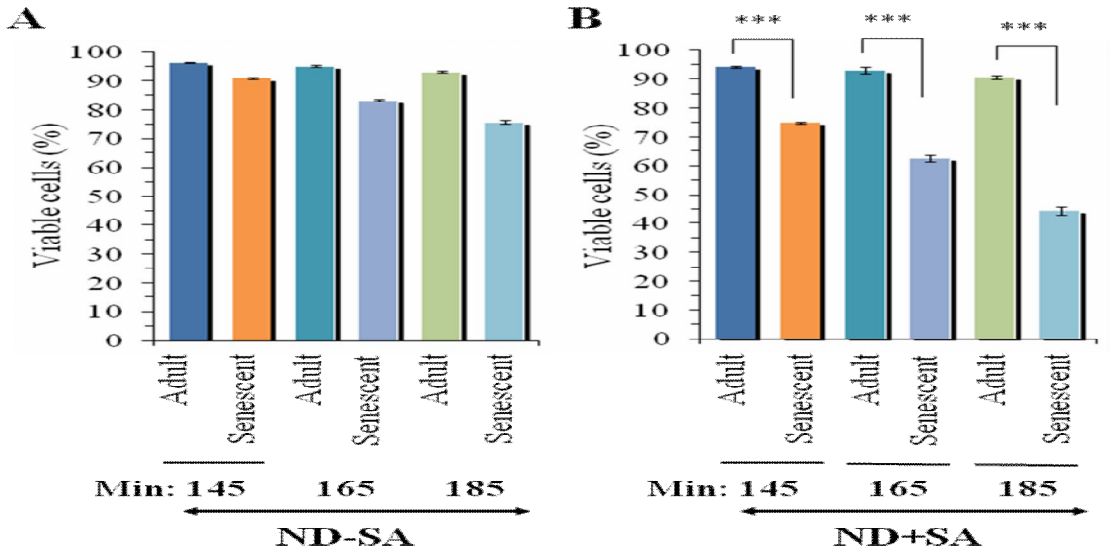


**Fig. 2** Senolytic activity of adult and senescent cells under nutrient deprived condition in the absence (ND-SA) or presence of sialic acid (ND+SA) as determined by cell proliferation assay. The data are representative of three independent experiments with S.D. indicated by error bars. *p* value <0.001 is indicated by three asterisks.

**Figure 3**

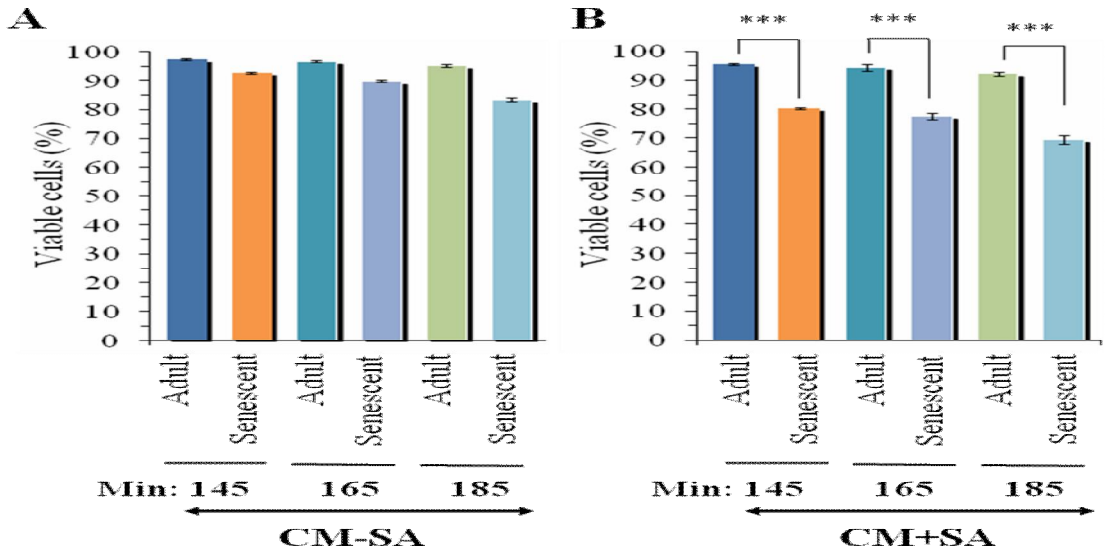


**Fig. 3** Senolytic activity of adult and senescent cells in complete medium in the absence (CM-SA) or presence of SA (CM+SA) as determined by cell proliferation assay. The data are representative of three independent experiments with S.D. indicated by error bars. *p* value <0.001 is indicated by three asterisks.

**Fig. 4A**

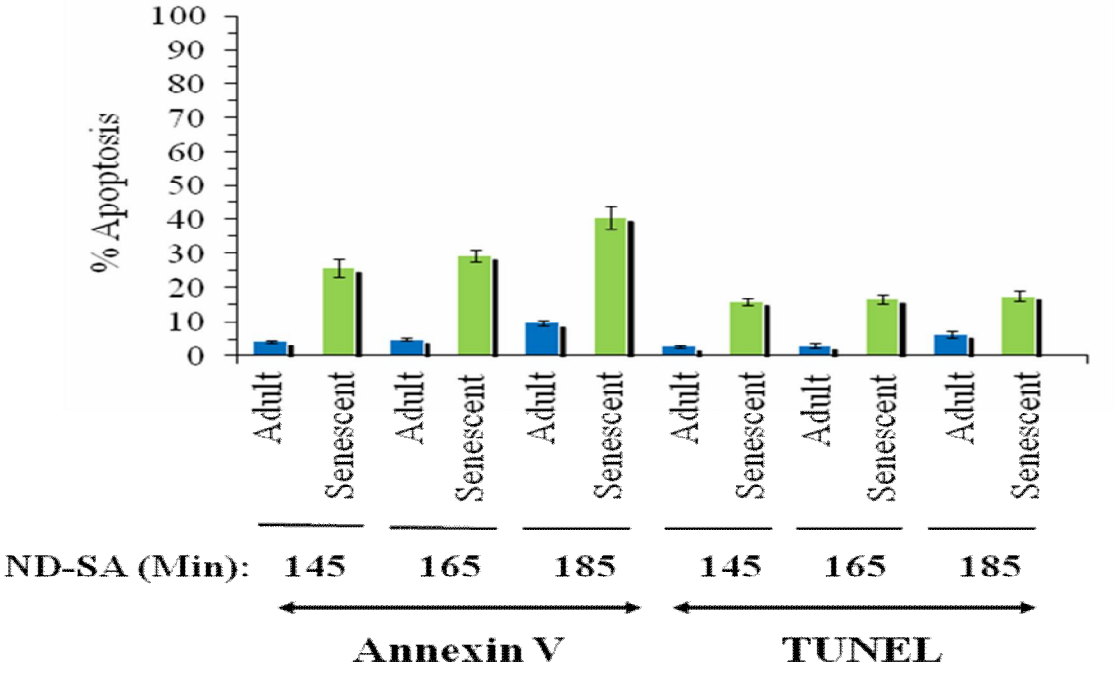

**Figure 4 B**

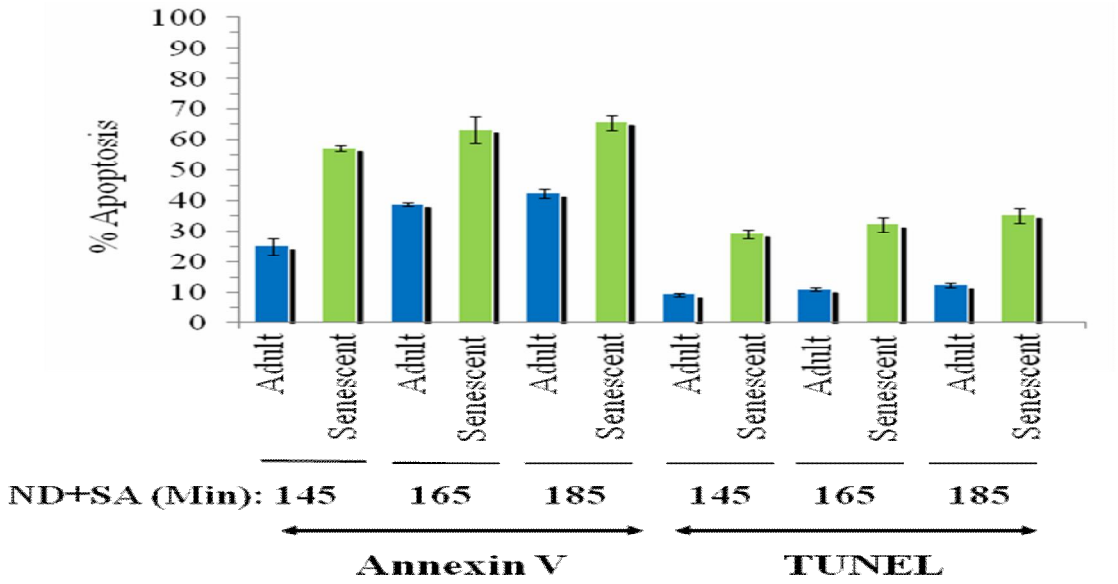


**Figure 4C**

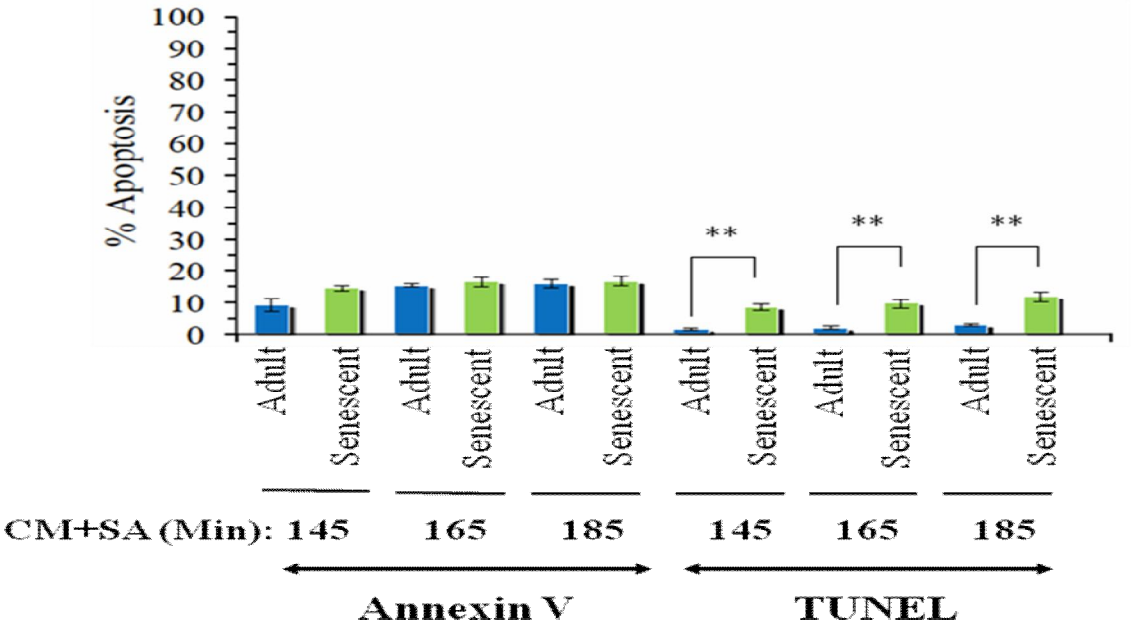


**Fig. 4** Apoptosis of adult and senescent LDE cells as demonstrated by Annexin V and TUNEL assays on a flow cytometer. Cells were treated in the absence or presence of 10 mM sialic acid under nutrient deprived condition (PBS) or in complete medium for 120 min followed by PBS wash (15 min) and additional PBS incubation (0, 20, and 40 min), and then stained with either Annexin V-FITC or FITC-labeled anti-BrdU mAb (TUNEL) (10 min) (total time: 145, 165, and 185 min), and analyzed by flow cytometry on FACSCalibur Flow Cytometer (Becton Dickinson Inc., Mountain View, CA). The data are representative of three independent experiments with S.D. indicated by error bars. *p* value <0.001 is indicated by three asterisks. **A.** ND-SA; **B.** ND+SA; **C.** CM+SA.

Figure 5A

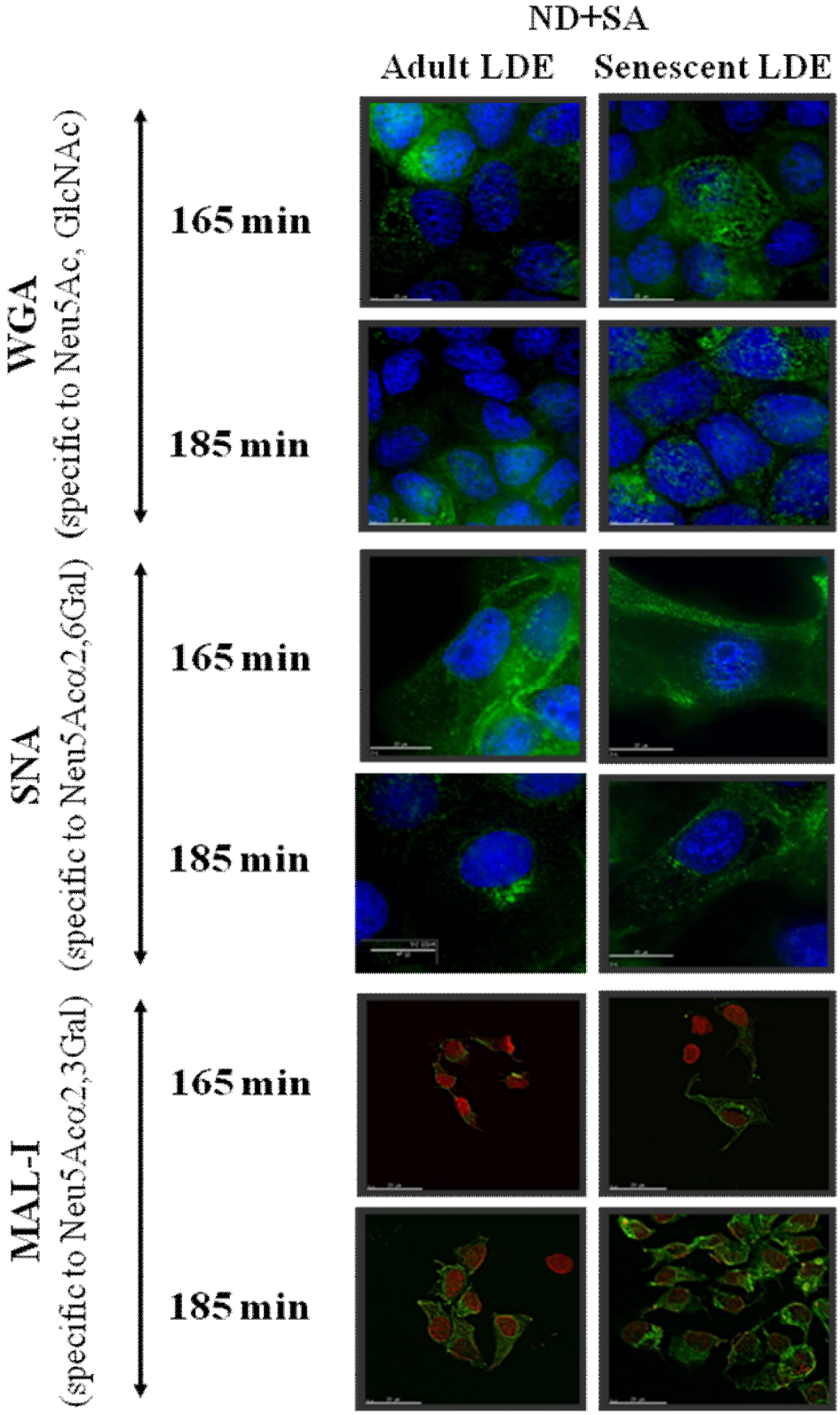
ND+SA
Adult LDE
Senescent LDE
WGA
(specific to Neu5Ac, GlcNAc)
165 min
185 min
SNA
(specific to Neu5Acα2,6Gal)
165 min
185 min
MAL-I
(specific to Neu5Acα2,3Gal)
165 min
185 min

**Figure 5B**

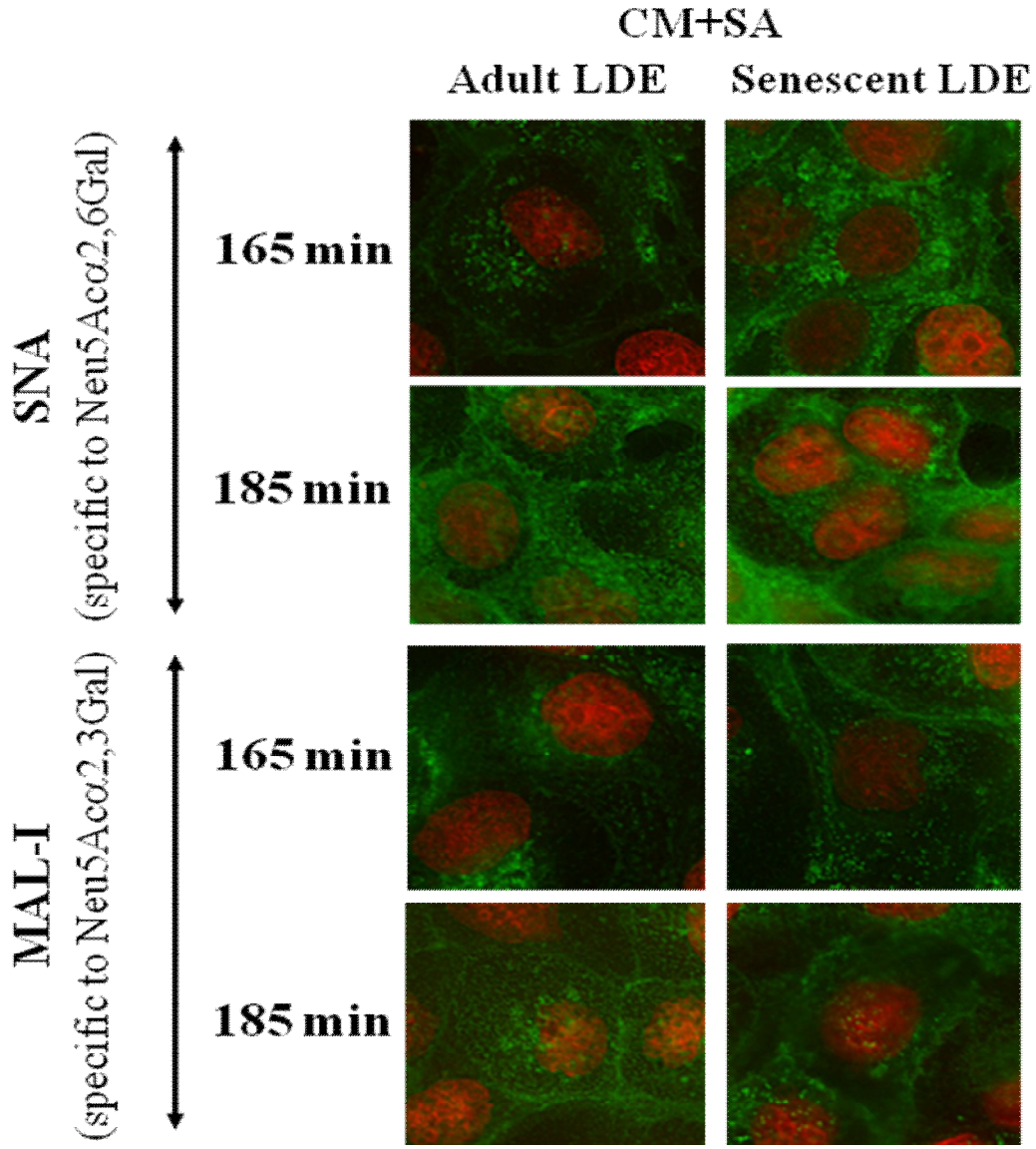


**Fig. 5** Lectin staining of live adult and senescent LDE cells as demonstrated by fluorescence microscopy. Cells were treated with 10 mM sialic acid in the nutrient deprived condition (PBS) or complete medium for 120 min and after very quick PBS wash or quick PBS wash plus additional PBS incubation (20 min), stained with lectin-FITC followed (5 µg/ml, 15 min, green fluorescence) by treatment with RNaseA (1 µg/ml, 15 min) and TO-PRO-3 (1 µM/ml, 15 min, blue fluorescence), Total time 165 min or 185 min. Fluorescence images were visualized at 63x Oil magnification and excitation were 365 nm, and 470 nm for blue and green fluorescence respectively. Images were captured on an advanced DeltaVision Elite imaging system and images were merged using softWoRx DMS from Applied Precision (Applied Precision Inc., USA). Merged images are shown at 15 µm (for WGA-FITC and SNA-FITC staining) and 20 µm magnification (for MAL-I-FITC staining). Neu5Ac, N-acetyl 5-neuraminic acid or sialic acid (SA); Gal, D-galactose; GlcNAc, N-acetyl D-glucosamine. **A.** ND+SA; **B.** CM+SA

## DISCUSSION

Dynamic changes in breast normal aging is thought to be associated with nutrient deprived environment due to vascular endothelial dysfunction and the decline in breast blood flow (Hartmann *et al.,* 2005). The LDE cells are important for both the structure and function of the human breast epithelium during lactation and represent the most potent derived cells in breast milk (Boutinaud and Jammes, 2002). The LDE cells can also be a good model for aging research as both adult and senescent cells can be easily obtained from breast milk of defined varying age groups. The assessment of apoptosis, which is a common phenomenon of normal aging, can also be easily performed in LDE cells.

Deletion of senescent cells increases the mice health and life span (Zhu *et al.,* 2015; Baker *et al.,* 2016; Schafer *et al.,* 2017). It has been shown conclusively in mice models that senescent cells maintained its survival by the upregulated function of transcription factor forkhead box protein O4 (Baar *et al.,* 2017). The effects of senescent cells and the SASP in fibrotic lung reveal that elimination of senescent cells and SASP blockade are novel anti-aging strategies for the treatment of later-stage diseases (Mercer *et al.,* 2015; Schafer *et al.,* 2017). Senescent cells expressed high levels of BCL-2 protein family, but undergo cell death when treated with senolytic compound (Zhu *et al.,* 2015; Schafer *et al.,* 2017). Similarly, sialic acid treatment under nutrient deprived conditions over 2 hours, as described here, efficiently killed senescent cells. Although the functional and molecular basis for its senolytic function remains to be elucidated, this would be particularly interesting for further glycobiology investigations especially how nutrient deprivation help senescent cells to make age-dependent tissue changes that promote cancer progression (Badr *et al.,* 2014; Badr *et al.,* 2017).

Taken together, this study is the first to show that SA supplementation can be protective to aging cells experiencing nutrient scarcity, while specifically killing senescent cells implying a possible role in anti-aging. Moreover, SA supplementation under nutrient deprived condition could be a strategic marker to distinguish senescent cells from adult or aging cells. However, further studies are needed to delineate the mechanism of senescent cell death and to examine the *in vivo* efficacy of SA supplementation under nutrient deprivation for anti-aging therapy.

**Acknowledgments**

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**Conflict of interest**

GlycoMantra, Inc. filed a non-provisional full patent entitled "Exploiting nutrient deprivation for modulation of glycosylation - research, diagnostic, therapeutic, and biotechnology applications" on August 16, 2016 with a priority date August 17, 2015. H.A. and H.A.B. are two of the three inventors. There are no other competing financial interests.

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