Eureka is Detroit Country Day’s scientific research journal. With this inaugural edition of Eureka, our aim is to celebrate and showcase the scientific pursuits of the students in the Science Research Club. This journal can be considered the culmination of efforts that began in DCD’s Middle School, when we first learned to navigate the scientific method of exploration, and our teachers planted in each of us the initial seeds of curiosity that would eventually bloom into a passion for scientific research. Our teachers in the Upper School and our project mentors fed this passion by their expertise and guidance. The vision and encouragement of Mr. Gene Menton, our faculty advisor, have made possible the publishing of this journal. As the word “eureka” literally means “I have found it”, one might ask, just what have we, the students who have written these papers, found? We have found a deeper understanding of the various research projects we conducted, projects ranging from mathematical proofs to cancer research. But more importantly, we have found out how much more there is to know, how many more questions there are to answer. We have found a whole new horizon of discovery, and an abiding love of inquiry.

Through this journal, we hope to inspire others to have their own “eureka moments” and find what we have found.

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Dexamethasone + Glutaric anhydride → Drug linker

{Chemical structures and images of cells}
Dendrimer-Dexamethasone Conjugates/Nanodevices for Targeted Delivery

Disha Bora

There are numerous eye diseases, some of which are minor and fleeting problems whereas others can lead to permanent vision loss. While common eye problems include cataracts, glaucoma, and retinal disorders, more serious diseases are age-related macular degeneration and diabetic retinopathy. For these ocular diseases, there is usually no cure, and often disease and loss of vision may progress despite treatment.

Polyamidoamine (PAMAM) dendrimers are repeatedly branched synthetic polymers with a well-defined spherical structure, tailored surface properties, nanometer scale size, a high degree of molecular uniformity, and the ability to deliver drugs intracellularly. Dendrimers start with a central initiator core, subsequently growing larger and more complex. With each new step, called a “generation” of polymer, their diameters increase and their molecular weight and number of surface functional groups double. Dendrimer properties are determined by functional groups on the molecular surface, as they make dendrimers hydrophilic and highly water soluble. Since these surface functional groups can be used to covalently attach imaging agents, drugs, or targeting ligands for targeted delivery or controlled release, dendrimers can be effective delivery vehicles. The purpose of small drug-dendrimer conjugates is to carry therapeutic agents to specific tissues in order to minimize systemic effects and increase efficacy at the targeted site. Conjugates need to be stable until they reach the targeted site. Then, they should release the drugs within the tissue before their removal from the area. Most small drugs are not active in conjugated form, so when conjugates are released early or are of high stability, the targeted delivery system fails. It is often challenging to prepare dendrimer-drug conjugates that can release the drugs within the target tissue in a predetermined manner. Understanding drug release profiles in conditions that these conjugates would exist in allows for a more accurate prediction of their in vivo efficacy. In turn, this greater understanding of the release mechanisms and profiles of PAMAM dendrimer-drug conjugates can help design effective delivery systems.

While linkages such as disulfide bonds have been previously shown, amide and ester bonds are most commonly used for the conjugation of small drugs to polymers\textsuperscript{1-3}. Previously, G5-PAMAM dendrimers conjugated to methotrexate (MTX) with an ester linkage were demonstrated to be stable in PBS buffer (pH = 7.4) for 2.5 hrs\textsuperscript{3}. In this study, dexamethasone, a synthetic member of the glucocorticoid class of steroid hormones and an anti-inflammatory and immunosuppressant drug, is conjugated to Generation 4 PAMAM dendrimers via ester linkages. The potency of dexamethasone is 20-30 times than that of hydrocortisone and 4-5 times than that of prednisone, both also steroidal drugs. Predominantly used to treat inflammatory and autoimmune conditions such as rheumatoid arthritis, it is also given to cancer patients undergoing chemotherapy to counteract certain side-effects of their antitumor treatment. Present in eye drops, dexamethasone has therapeutic uses related to endocrine, obstetrics, and high altitude illness, as well as cerebral and pulmonary edema. Dexamethasone decreases inflammation by suppression of neutrophil migration, decreased production of inflammatory mediators, and decreased capillary permeability. It also suppresses normal immune response. For long term therapy, a dosage of 0.5 to 1.5 mg oral per day is recommended. Since dexamethasone is water insoluble and drugs can be covalently coupled onto the surface of the dendrimer, dexamethasone can readily conjugate with the dendrimer\textsuperscript{3}. Dexamethasone was the drug of choice for its ability to conjugate with PAMAM dendrimers and because of its presence in eye
drops, making it a more significant drug for synthesis of a conjugate to treat relevant ocular diseases (Figure 1).

This study seeks to develop and characterize a dendrimer-dexamethasone conjugate to improve therapy for relevant ocular diseases, and then understand its release profile. Combining the targeting capability of PAMAM dendrimers with a better understanding of their release characteristics will improve the ability to design high-efficacy conjugates for ocular diseases.

Materials
Generation four hydroxyl terminated PAMAM dendrimer (G4-OH) in methanol solution was purchased from Dendritech Inc (Midland, MI, USA). Dexamethasone, 6(FMOC-amino)caproic acid, (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), N,N-Diisopropylethylamine (DIEA), piperidine, glutaric anhydride, N,N-dimethylacetamide (DMA), N-Hydroxybenzotriazole (HOBt), and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Triethylamine (TEA) from Fisher and trifluoroacetic acid (TFA), dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) from EMD were purchased. All anhydrous solvents DMSO, DMF, acetonitrile (ACN), and dichloromethane (DCM)) were purchased from Acros Organics (Morris Plains, NJ). All other solvents and chemicals used were purchased from Fisher Scientific (Waltham, MA). Regenerated cellulose (RC) dialysis membrane with molecular weight cut-off of 1000 Da was obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA).

Methods
Characterization
All reactions were carried out under nitrogen conditions. The anhydrous solvents and other reagents that are commercially available were directly used without further purification. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian INOVA 400 spectrometer using commercially available deuterated solvents. Proton chemical shifts were reported in ppm (δ) and tetramethylsilane (TMS) used for internal standard. Coupling constants (J) were reported in hertz (Hz). Thin layer chromatography (TLC) was performed on silica gel GF254 plates (Watmann) and the spots were visualized with UV light and 2% H2SO4 in EtOH. Mass spectra were recorded on Waters Micromass ZQ spectrometer. MALDI-TOF spectra were recorded on a Bruker Ultraflex system equipped with a pulsed nitrogen laser (337 nm), operating in positive ion reflector mode, using 19 kV acceleration voltage and a matrix of 2,5-dihydroxybenzoic acid.

Synthesis of Bifunctional G4 PAMAM dendrimer
PyBOP (547 mg, 1.05 mmole) and DIEA (163 mg, 1.26 mmole) each dissolved in DMSO (5 mL) were added to a stirring solution of 6(FMOC-amino)caproic acid (371 mg, 1.05 mmole) in DMSO (10 mL), under nitrogen atmosphere. The mixture was allowed stir for 1 hr at room temperature and PAMAM G4-OH (598.8 mg, 0.0420 mmole) dissolved in DMSO (5 mL) was added to the reaction mixture. The reaction was carried out for 48 hrs at room temperature. The resulting solution was dialyzed extensively with DMSO (dialysis membrane of molecular
weight cutoff = 1000 Da) for 24 hrs and deionized water for 6 hrs. The obtained reaction mixture was then lyophilized yielding 539 mg of intermediate. The intermediate contains FMOC-protected amino groups on the surface. NMR was performed to determine the number of FMOC-protected amino groups and later to confirm that the deprotection of the FMOC group was successful. To obtain free amine groups, 4 mL of piperidine and 16 mL of DMF (Piperidine:DMF ratio was 2:8) was added to G4-OH conjugate containing FMOC groups. The reaction was carried out for 1 h under nitrogen atmosphere, and then the solvent was removed by evaporation at reduced pressure. The mixture was redissolved in DMSO and dialyzed with DMSO for 24 hrs and deionized water for 6 hrs. The reaction mixture was then lyophilized yielding 414 mg of bifunctional dendrimer.

**Synthesis of Dexamethasone-2'-glutarate**

Glutaric anhydride (174.434 mg, 1.529 mmole) and TEA (35.5 μL) were added to a solution of dexamethasone (200 mg, 0.510 mmole) in 80:20 (v/v) anhydrous DMA/DMF (5 mL). The reaction mixture was stirred for 24 hrs under N₂, while being monitored with TLC. After 3 days of stirring, TLC (methanol/ethyl acetate/DCM = 5:2:2, Rf = 0.28) showed that the reaction was complete. The reaction mixture was evaporated under reduced pressure and pure dexamethasone-2'-glutarate was isolated by flash column chromatography on silica gel using methanol:ethyl acetate:hexanes = 5:2:2 as mobile phase. NMR spectra for the drug and the drug-linker were performed and recorded.

**Dendrimer-Dexamethasone Conjugate**

To a stirring solution of bifunctional dendrimer (240 mg, 0.0153 mmole) in anhydrous DMSO, TEA (41 μL) and then dexamethasone-2'-glutarate (77.5 mg, 0.153 mmole) dissolved in anhydrous DMSO was added. The reaction mixture was allowed to stir for 48 hrs at room temperature under nitrogen conditions. After 24 hrs, EDC.HCl (43.9 mg) and HOBt (30.9 mg) dissolved in DMF (3 mL) was added. Once the reaction was complete, the reaction mixture was subjected to dialysis in DMSO (membrane MW cutoff = 1000 Da) for 24 hrs. The obtained reaction mixture was placed in an evaporator to remove excess DMF and then lyophilized to get the final dendrimer-dexamethasone conjugate. A final NMR of the conjugate was recorded.

**HPLC characterization**

HPLC characterization was carried using Waters HPLC instrument equipped with dual pumps, an auto-sampler and dual UV detector interfaced to Breeze software. The HPLC chromatogram was monitored at 242 nm using the dual UV absorbance detector. H₂O:ACN (0.14% TFA) was used as mobile phase. HPLC analysis was done using 90:10 to 30:70 (H₂O:ACN) gradient flow in 30 minutes with flow rate of 1 mL/min. The release studies were performed in 0.1 M PBS buffer solution (pH = 7.4). The drug payload, or the % drug in 1 mL buffer, was ~13%. The conjugate was added into 3 mL preheated buffer solutions in triplicates. All the release solutions containing 1 mg/mL dendrimer drug conjugate dissolved in PBS were stirred continuously and maintained at 37°C. At appropriate time intervals, samples were withdrawn and immediately analyzed by HPLC to determine the EM concentrations.
Results

**Synthesis of Bifunctional G4 PAMAM dendrimer**

$^1$H NMR (DMSO-$d_6$) δ 1.21 (m, CH$_2$ protons, linker), 1.36 (m, CH$_2$ protons, linker), 1.47 (m, CH$_2$ protons, linker), 2.18 (bs, CH$_2$ protons of G4-OH and COCH$_2$ protons of linker), 2.41 (bs, CH$_2$ protons, G4-OH), 2.62 (bs, CH$_2$ protons, G4-OH), 2.92-2.94 (m, CH$_2$NH protons, linker), 3.03-3.11 (m, CH$_2$ protons, G4-OH), 3.25 (m, CH$_2$ protons, G4-OH), 3.35-3.38 (t, CH$_2$ protons, G4-OH), 3.97 (m, CH$_2$OC=O protons, G4-OH), 4.17 (m, CH proton of FMOC group), 4.25-4.27 (m, OCH$_2$ protons of FMOC group), 4.69 (bs, OH, G4-OH), 7.23 (m, NHCOO, carbamate protons), 7.28-7.30 (t, aromatic protons, FMOC), 7.36-7.39 (t, aromatic protons, FMOC), 7.64-7.65 (d, aromatic protons, FMOC), 7.76-8.02 (m, aromatic protons of FMOC and amide protons of G4-OH).

Since amine groups have better reactivity compared to hydroxyl groups, a bifunctional dendrimer was prepared (Scheme 1). 6(FMOC-amino)caproic acid was used as a linker to provide protected NH$_2$ groups. Generation 4 hydroxyl terminated PAMAM dendrimer (G4-OH) was reacted with activated 6(FMOC-amino)caproic acid by PyBOP. The intermediate was confirmed by proton NMR chemical shift and integration of FMOC protected groups. There were also chemical shifts of methylenes that belong to the linker and dendrimer ester methylenes that appear after conjugation. The integration of a characteristic peak for FMOC groups obtained from NMR suggested that 13 molecules of FMOC-linker were conjugated to the dendrimer.

**Deprotection of FMOC group**

$^1$H NMR (DMSO-$d_6$) δ 1.24 (m, CH$_2$ protons, linker), 1.34 (m, CH$_2$ protons, linker), 1.48 (m, CH$_2$ protons, linker), 2.18-2.27 (m, CH$_2$ protons of G4-OH and COCH$_2$ protons of linker), 2.41 (bs, CH$_2$ protons, G4-OH), 2.62 (bs, CH$_2$ protons, G4-OH), 3.07-3.10 (m, CH$_2$ protons, G4-OH), 3.25 (m, CH$_2$NH protons, linker), 3.36-3.39 (t, CH$_2$ protons, G4-OH), 3.98 (t, CH$_2$OC=O protons, G4-OH), 7.78-8.02 (m, amide protons of G4-OH).

The deprotection of FMOC-protected amino groups using piperidine/DMF (2:8), to preserve the dendrimer by obtaining free amine groups gave the bifunctional dendrimer 50 OH and 14 NH$_2$ groups.

**Synthesis of Dexamethasone-21-glutarate**

$^1$H NMR (DMSO-$d_6$) δ 0.76-0.77 (d, 3H, -CH$_3$), 0.86 (s, 3H, -CH$_3$), 1.02-1.07 (m, 1H, -CH), 1.27-1.37 (m, 1H, -CH), 1.47 (s, 3H, -CH$_3$), 1.54-1.62 (m, 2H, -CH$_2$), 1.71-1.78 (m, 2H, -CH$_2$ protons of linker), 2.06-2.16 (m, 2H, -CH$_2$), 2.26-2.37 (m, 4H, -CH$_2$ protons of linker and -CH$_2$ protons of dexamethasone), 2.38-2.48 (m, 2H, -CH$_2$ protons of linker), 2.57-2.62 (m, 1H, -CH), 2.84-2.87 (m, 1H, -CH), 4.11-4.14 (d, 1H, -CH), 4.76-4.78 (d, 1H, CH), 4.99-5.03 (d, 1H, CH), 5.13 (s, 1H, OH proton), 5.38 (s, 1H, CH), 5.98 (s, 1H, aromatic proton), 6.19-6.22 (dd, 1H, aromatic proton), 7.26-7.28 (d, 1H, aromatic proton), 12.13 (bs, 1H, COOH proton).

The reaction of dexamethasone with glutaric anhydride in the presence of triethylamine occurred at the position 21 which gave dexamethasone-21-glutarate (Scheme 2). The compound was characterized by proton NMR and HPLC. The NMR spectra recorded confirmed the formation of the dexamethasone-linker compound.
Synthesis of Dendrimer-Dexamethasone Conjugate

$^1$H NMR (DMSO-$d_6$) δ 0.76-0.78 (d, methyl protons of Dex), 0.87 (s, methyl protons of Dexa.), 1.03-1.08 (m, CH proton of Dexa.), 1.22 (m, -CH$_2$ protons of linker), 1.36 (m, -CH$_2$ protons of linker), 1.44-1.63 (m, methyl and -CH$_2$ protons of Dexa. and -CH$_2$ protons of linker), 1.73-1.78 (m, and -CH$_2$ protons of linker), 2.09-3.39 (m, CH$_2$ protons of G4-OH, protons of Dexa and -CH$_2$ protons of linker), 3.98 (t, CH$_2$OC=O protons, G4-OH), 4.13 (m, CH protons of Dexa.), 4.69 (bs, OH protons of G4-OH), 4.75-4.80 (d, CH protons of Dexa.), 4.99-5.03 (d, CH protons of Dexa.), 5.13 (s, OH protons of Dexa.), 5.38-5.39 (d, CH protons of Dexa.), 5.99 (s, aromatic protons of Dexa.), 6.20-6.22 (d, aromatic protons of Dexa.), 7.27-7.29 (d, aromatic protons of Dexa.), 7.76-8.03 (m, amide protons of G4-OH).

The NMR spectra indicate a dendrimer-dexamethasone conjugate was formed (Scheme 3).

Release studies

The drug release rate of the conjugate was analyzed in PBS buffer, as the conjugate (1 mg/mL) was soluble in this buffer. Calibration was at 100 µg/mL with a 6,000,000 peak area at 242 nm. The ester linkage used for conjugation of the dexamethasone was liable to hydrolysis. In 7 days, about 37.5% of dexamethasone was released (Figure 2). This represents a drug payload in the conjugate of ~13%. Release studies are still ongoing and further analysis is needed. However, this release profile should prove suitable for the present application, as anti-inflammatory activity is desired over a short period of time. In fact, it is hoped that the dendrimer will serve as a vehicle for the drug by delivering and releasing it inside cells in a predetermined manner or over a period of several hours.

Conclusion

A PAMAM dendrimer-dexamethasone conjugate was synthesized, characterized, and evaluated for its release properties. Hydroxyl-terminated poly(amidoamine) dendrimer (G4-OH) was reacted to a protected amine linker followed by deprotection to obtain a bifunctional dendrimer that contained 14 free amine groups. Dexamethasone was modified by incorporation of glutaric acid and then conjugated to the bifunctional dendrimer. The conjugate was characterized by $^1$H NMR, MALDI and HPLC. Release studies are ongoing so the conjugate’s release profile is not completely understood at this point. However, current results indicate a 13% drug payload, as 37.5% of the drug was released in a period of 7 days.

It is hoped that dendrimer-dexamethasone conjugates prove an effective treatment for ocular diseases. Detailed analysis of release studies will enable better synthesis of a conjugate that can release at a predetermined site of action in the eye. Further studies will focus on determining whether dendrimer-dexamethasone can generate similar or better effects in vivo with the use of a mouse model.
Figures and Schemes

Figure 1. Dexamethasone

Scheme 1. Synthesis of bifunctional PAMAM dendrimer

Scheme 2. Synthesis of Dexamethasone-2’-glutarate
Scheme 3. Synthesis of Dendrimer-Dexamethasone Conjugate

Figure 2. Percent Dexamethasone Released over 7 days
References


The Effects of Epidermal Growth Factor (EGF)-like Growth Factor Ligands on EGF-Receptor (EGFR) Plasma Membrane Accumulation in SUM-149 Inflammatory Breast Cancer Cells

Hannah Cheriyan

Terms
Plasma membrane: The outer layer of a cell; is semi-permeable and contains various proteins
Receptor: Type of protein on cells’ plasma membranes to which signaling molecules called ligands attach
Ligand: Protein that binds (attaches) to a receptor to facilitate a cellular process, i.e. metabolism
Concentration: The amount of a substance per unit of liquid that the substance is dissolved in; the higher the concentration, the more of the substance is added
Intracellular: Inside a cell
Endosomes: Small membrane-enclosed bodies contained in cells; used to store molecules
Lysosomes: Small membrane-enclosed bodies contained in cells; break down molecules
Medium: Combination of nutrients in which cells grow in an in vitro lab setup; plural is media
In vitro: Literally “in glass”; means experiments not conducted in the body, but instead conducted on Petri dishes or other artificial structures
(Cell) Culture: The process of growing and maintaining cells in vitro
Seeding: The process of placing a relatively small number of cells on a Petri dish (or “plate”), letting the cells attach to the surface, and allowing them to multiply
Confluence: When plated cells have multiplied so that they cover the surface they grow on while staying connected to each other; also known as “100% confluence”, thus “50% confluence” is when cells spread out, so that they are not so connected to each other, and cover about half of the surface
Epidermal Growth Factor Receptor (EGFR) is a transmembrane receptor tyrosine kinase that is often found in increased amounts on the plasma membranes of malignant epithelial tumors. The various ligands that can bind to EGFR include epidermal growth factor (EGF), amphiregulin (AREG), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor α (TGF-α), betacellulin (BTC), epigen (EPGN), and epiregulin (EREG). The effects of high concentrations of EGF on EGFR are well known—EGF induces EGFR to sink from the cell membrane into the intracellular endosomes of the cell, to be degraded by lysosomes after about an hour. However, the effects of lower concentrations of EGF and the effect of other ligands on EGFR membrane accumulation have been less studied.

Previous studies of AREG have shown that levels of AREG expression are generally higher in invasive breast cancers than in nonmalignant tumors, and that there is a correlation between breast cancer’s aggressiveness and the levels of AREG expression in the cells. Previous experimentation also demonstrated that the inflammatory breast cancer cell line SUM-149 has a functioning AREG/EGFR autocrine loop (a mechanism whereby a cell self-produces AREG, and consequently self-regulates its own EGFR production), which enhances the cells’ proliferation, motility and invasive capacity and seems to effectively prevent EGFR internalization and degradation so that EGFR accumulates on the outside of the cells’ plasma membranes.

SUM-149 knock-down cell lines sh4 and shPlc, lines in which the endogenous AREG expression of SUM-149 is blocked using shRNA (short hairpin RNA) interference, were created. As the membrane-accumulation characteristics of EGFR in an AREG-rich environment were well known, it was decided to investigate the effects of various concentrations of EGF on the growth and EGFR membrane accumulation of these AREG knock-down cells, and also test the effects of the other EGF-like ligands, barring AREG, on the growth and EGFR membrane accumulation in the knock-down cells, in order to better understand the possible crucial implications of EGFR plasma membrane accumulation. The purpose of this work was to test the hypothesis that the other EGF-like ligands would not have a significant effect on cell growth and EGFR plasma membrane accumulation, but decreased concentrations of EGF would increase cell growth and increase EGFR membrane accumulation to a point where EGFR membrane accumulation in the cells would be equal to EGFR internalization in the cells.

Procedure

Cell Culture

Cell lines used in this experiment were SUM-149, SUM-149sh4, SUM-149shPlc (both AREG knock-downs), and SUM-149shNS (which served as the specific control for the knockdown cell lines, as it contains shRNA as well, although its shRNA does not block endogenous AREG expression). Cells were cultured in 6-well plates. The base medium the cells were maintained in was Ham’s F-12 with 5% Fetal Bovine Serum (FBS), 5µg/ml insulin, 2µg/ml hydrocortisone, 5µg/ml gentamicin (antibacterial agent), and 2.5µg/ml fungizone (antifungal agent)—medium also known as 5%IH. SUM-149 and SUM-149shNS, as control cells wherein AREG expression occurred, were always grown only in 5%IH or 5%IH + puromycin (P). SUM-149sh4 and shPlc each had three wells of cells growing in one of ten different kinds of media, all with base 5%IH+P: the base itself, base+10ng/ml EGF, base+1ng/ml EGF, base+0.3ng/ml EGF,
base+0.1ng/ml EGF, base+10ng/ml EREG, base+10ng/ml TGF-α, base+10ng/ml BTC, base+10ng/ml EPGN, or base+10ng/ml HB-EGF. All cells were maintained in a humidified incubator at 37°C and 10% CO₂. The cells were reseeded in their media when they reached confluence.

**Immunofluorescence Assay**

After growing the cells in their respective media for 6 days, the cells were sub-cultured and reseeded on coverslips. 8 days later they reached 50% confluence on the coverslips, so the cells were rinsed with phosphate buffered saline (PBS) solution, agitated in 1ml of 4% para-formaldehyde for 20 minutes to fix them, rinsed in PBS, and placed on ice in 1ml of 0.1% Triton-X in PBS for 2 minutes. They were then rinsed in PBS and agitated in 1ml of 20% goat serum in PBS for 1 hour. A 1:25 dilution of anti-EGFR antibody was prepared, and the cells were incubated on parafilm with 30 µl of the antibody per coverslip for 1 hour. The cells were rinsed with PBS, then incubated with an Alexa488-conjugated anti-mouse secondary antibody (Molecular Probes) for 1 hour in the dark. After this, the cells were rinsed first with PBS and then with distilled water. ProLong gold with DAPI was used as a mounting agent (Molecular Probes), and coverslips were glued on slides to prepare them for photo-microscopy.

**Western Blot**

After growing the cells in their respective media for 21 days, the cells were lysed in a buffer of 20 mM Tris-HCl, 137 mM NaCl, 1% NP40, 10% glycerol, 1mM Na₃VO₄, 1mM phenylmethylsulfonyl fluoride, 1% aprotinin, and 20 µg/ml leupeptin. Protein concentrations were found using a Bradford Protein Assay. Sample buffer was added and the samples of lysed cells were boiled for five minutes in a sand bath, after which the amount of sample to be loaded was calculated by dividing 50µg, the desired amount of protein per lane, by the protein concentration of each sample that had been determined by Bradford Protein Assay. The gel apparatus was then assembled using 7.5% Bis:Acryl mini-gels prepared in advance. 5µl of marker and 5µl of sample buffer were mixed and loaded into the first lane of the first gel, and the subsequent lanes were loaded with the previously calculated amounts of each sample. The apparatus was run for 1.5 hours at 20 mA, after which the gels were removed, soaked in dry transfer buffer for 15 minutes, and placed into the dry transfer apparatus on top of membranes soaked in the same buffer and covered by blotting paper. The apparatus was run for 1 hour at 20 V. The membranes were then removed from the apparatus and blocked for an hour in 5% BSA in TTBS, then blocked overnight in mouse monoclonal anti-EGFR antibody clone 31G7 (Zymed Laboratories, Inc.) and for another hour in anti-phosphotyrosine antibody PY-20 (Calbiochem). The resulting blots were visualized by film and enhanced chemiluminescence (ECL).

**Growth Assay**

After growing the cells in the different concentrations of EGF for 22 days, the cells were plated after being counted with a hemacytometer, to ensure equal seeding in each well plate. Six wells of each of the SUM-149shPld and sh4 cell types growing in EGF and six wells of SUM-149 and SUM-149shNS were plated to be counted in duplicate after 1, 4, and 7 days. On the appropriate days, two wells of each cell line were emptied of media, rinsed with PBS, agitated for five minutes in 500µl of Hepes solution, agitated for ten minutes after 75µl of Bretol solution was added, and the contents of each well were pipetted into Coulter Counter cups. The cups were inserted into the Coulter Counter and the cell count was recorded. This procedure was repeated with cells growing in the other EGF-like ligands after they had been growing for 27 days.
Data

**Immunofluorescence Assay**
All green staining represents EGFR; all blue staining represents the cell nuclei.

**Cells Grown in Various Concentrations of EGF**

**Figure 1**
SUM-149sh4 in 10ng/ml EGF
Note small, intracellular points of EGFR (green)

**Figure 2**
SUM-149sh4 in 1ng/ml EGF
Now EGFR is less punctate (point-like) and begins to cover the plasma membrane

**Figure 3**
SUM-149sh4 in 0.3ng/ml EGF
With decreased EGF concentration, there is more EGFR on the outside of the cell

**Figure 4**
SUM-149shPld in 10ng/ml EGF
Very bright, concentrated points of EGFR near cell nuclei

**Figure 5**
SUM-149shPld in 1ng/ml EGF
EGFR is still intracellular, but moving away from cell nuclei

**Figure 6**
SUM-149shPld in 0.3ng/ml EGF
Least concentration of EGF again yields bright EGFR all over plasma membranes

**Cells Grown in 10ng/ml Concentrations of Various EGF-Family Ligands**

**Figure 7**
SUM-149sh4 in 10ng/ml BTC
Bright points of EGFR near cell nuclei- BTC seems to act like EGF

**Figure 8**
SUM-149sh4 in 10ng/ml EREG
EGFR covering cell plasma membranes- brightest near nucleus and where cells join (cell-cell junctions)
Cells Grown in Base Media (no added ligands)

**Figure 9**
SUM-149sh4 in 5%IH+P (base media)
In these knock-downs (no endogenous AREG expression), EGFR covered plasma membranes; cells grew in small groups of two or three, or individually.

**Figure 10**
SUM-149shPld in 10ng/ml BTC
Bright points of EGFR near cell nuclei

**Figure 11**
SUM-149shPld in 10ng/ml EREG
EGFR covering cell plasma membranes

**Figure 12**
SUM-149shPld in 5%IH+P (base media)
EGFR covered cell plasma membranes in these knockdowns as well- like the sh4 cells, these grew in small groups or individually.

**Figure 13**
SUM-149 in 5%IH(base media)
In these control cells that express AREG, EGFR covers the plasma membranes; spiky projections connecting cells are especially bright, and cells grew in sheets- large, connected groups

**Figure 14**
SUM-149shNS in 5%IH+P (base media)
In these AREG-expressing control cells, the plasma membranes are again covered by EGFR; cell-cell junctions are bright, and cells grew in sheet-like formation, like the normal SUM-149 cells
Western Blot

Figure 15
This blot gives two pieces of information about each cell line- the first row (EGFR) shows how much EGFR protein was detected in each cell line, and the third row (P-Tyr) presents the amount of a certain phosphotyrosine protein detected in each cell line, which shows how much the EGFR is being phosphorylated, or activated. The second and fourth rows show how much solution was initially loaded into each gel. Note that the amount of P-Tyr remains approximately constant in every cell line, except SUM-149shPld growing in base media (#14 in blot), which seems to have more P-Tyr than the other lines. However, the amount of EGFR in each cell line varies greatly, with SUM-149sh4 in 10ng/ml EGF and in 10ng/ml BTC (#14 and #11) and SUM-149shPld in the same ligands (#15 and #22) having the least EGFR detected, while the most EGFR was detected in SUM-149sh4 in 0.1ng/ml EGF (#8) and SUM-149shPld in base media and in 0.1ng/ml EGF (#14 and #19). Thus, although growing cells in the lowest concentration of EGF yielded the most detected EGFR, and growing cells in 10ng/ml of BTC yielded nearly imperceptible amounts of EGFR, results also seen in the immunofluorescence assay, according to the similar levels of P-Tyr in each cell line, EGFR is still being activated at the same level even when it is undetectable.
**Growth Assay**

*Figure 16*

These graphs show how many cells had grown in each cell line after a certain number of days, with each colored line representing a different cell line. In both assays, after 7 days the SUM-149 and SUM-149shNS cell lines had the highest cell count, and there was a significant gap between their end number of cells and the end number of cells in the other cell lines. In the first assay, the cell lines with the lowest end cell count and thus the slowest rate of growth were the cell lines growing in 10ng/ml EGF, a result consistent with the data from the Western Blot and immunofluorescence assay.
Results and Discussion

Immunofluorescence Assay

An immunofluorescence assay was performed on the cells in order to compare the amount and location of EGFR in the cells. Pictures were taken of every cell line. From the pictures taken, it was observed that the lower the concentration of EGF that cells were growing in, the more EGFR accumulated on their plasma membranes. Conversely, the higher the concentration of EGF the cells grew in, the more intracellular the location of the EGFR in the cells and the more point-like its appearance. Previous studies have determined that EGF stimulation sends EGFR from the plasma membrane and into endosomes\textsuperscript{3}, and the observation of the intracellular points of EGFR upon EGF stimulation seems to be confirmed by these studies. Interestingly, it was also observed that the brightness of the green-stained EGFR was highest at cell-cell junctions on the membrane and in the intracellular points around the cell nuclei induced by high EGF concentrations. This brightness suggests that the amount or activity of EGFR was greatest in these areas. The most relevant results pertaining to the cells growing in ligands other than EGF were that cells growing in 10ng/ml BTC displayed the same characteristics as cells growing in high concentrations of EGF: bright, intracellular points of EGFR located very close to the nuclei. On the other hand, cells growing in 10ng/ml EREG and 10ng/ml EPGN had the largest amount of EGFR on their plasma membranes, amounts comparable to those of cells growing in the lowest concentrations of EGF or in the presence of AREG. The controls for this assay were the endogenous AREG-expressing cell lines SUM-149 and SUM-149shNS, which both, being grown in the base media without any extra ligands added, acted according to expectation and displayed large amounts of EGFR on their plasma membranes.

Unexpectedly, it was observed that the amounts of EGFR on the plasma membranes of SUM-149sh4 and shPld cells grown in the base media were nearly equal to the amounts of membrane-EGFR on the control cells, whereas it had previously been thought that the control cells would have much more EGFR on the membrane as they still had functioning AREG autocrine loops. The main difference between the control cells and the SUM-149sh4 and shPld cells was that the control cells grew in a more confluent and sheet-like fashion, with large groups of cells that were all connected to each other, while the SUM-149sh4 and shPld cells were more spread out and separate from each other, with small groups of only two or three cells connected to each other (See Data: Immunofluorescence Assay: Cells Grown in Base Media for pictures).

Western Blot

A Western Blot of the cells was run to compare the cells’ EGFR protein expression and their EGFR tyrosine phosphorylation. It was subsequently observed that out of the cells growing in different concentrations of EGF, EGFR protein expression was the highest in SUM-149sh4 and shPld cells growing in 0.1ng/ml of EGF, the least concentration of EGF used in the experiment- a result consistent with the immunofluorescence assay performed earlier. However, the levels of tyrosine phosphorylation were approximately equal in all cell lines except for SUM-149shPld in base media and SUM149-shPld in 0.1ng/ml EGF, showing that even where EGFR expression was not detected, EGFR was still being activated and phosphorylated. This result could be explained by postulating that if the internalized EGFR did, in fact, enter the cells’ endosomes and was not degraded immediately, it could have been undetectable by Western Blot yet still active. Furthermore, the fact that EGFR was still being activated inside the cells means
that any metabolic pathways utilizing EGFR were most likely still functional. Thus, high concentrations of EGF (or of BTC) that instigate the internalization of EGFR can be said to stunt only cell growth, not cell function. This ponderous conjecture was not addressed further during this experiment- however, it may be the subject of later tests.

The salient points of the blots of the cells growing in the various ligands were that a higher level of EGFR expression was observed in SUM-149sh4 and shPld cells growing in 10ng/ml EREG and 10ng/ml EPGN than in the other cell lines, and a significantly lower level of EGFR expression was observed in SUM-149 sh4 and shPld cells growing in 10ng/ml BTC, results again consistent with the immunofluorescence assay. The controls used in this blot were the cell lines SUM-149 and SUM-149shNS, and although the SUM-149 lane of the gel was not loaded equally and thus did not yield expected results, the SUM-149shNS cell line acted expectedly and displayed high EGFR expression.

**Growth Assays**

In order to determine the effect of EGF and the other ligands on cell growth rate, two growth assays were performed: one assay of the cells growing in EGF with SUM-149 and SUM-149shNS as controls, and one assay of the cells growing in other ligands with SUM-149 and SUM-149shNS as controls again. The control cells acted expectedly in both assays. The results of the growth assay of the cells growing in EGF showed that the lower the concentration of EGF, the more growth the cells attained. There was also a significant gap between the end cell population of the control cells and of the cells growing in EGF, suggesting either that AREG highly enhances cell growth or that EGF even in small amounts stunts cell growth significantly. It is more likely that AREG highly enhances cell growth, as the gap between the end cell count of the control cells and the variable cells was evinced in the growth assay of the cells growing in other ligands as well. Besides the fact that the control cells grew much more than the cells growing in the other ligands, it was noted that out of the other cells those growing in 10ng/ml EPGN seemed to grow the most. A difference in the growth rates of SUM-149shPld and SUM-149sh4 cells was also observed, as the shPld cells seemed on average to grow more than the sh4 cells, a difference that had been noticed in the previous weeks of cell culture.

**Conclusion**

The results overall answer my questions and support my hypothesis that in decreased concentrations of EGF, SUM-149sh4 and shPld growth would increase and EGFR would be found increasingly on cells’ plasma membranes. However, as according to the tyrosine phosphate blots EGFR is still being phosphorylated even in the highest EGF concentrations, high EGF concentrations only stunt cell growth, not cell function. This essentially eliminates EGF as a possible chemotherapeutic agent, since EGF does not affect EGFR activation. On the other hand, the results have raised new questions about the nature of AREG and EGF cellular interactions. Previous publications noted that in SUM-149 breast cancer cells, the AREG autocrine loop enhanced EGFR membrane accumulation. It was thus expected that the amount of EGFR on the membranes would be less in the AREG knock-down cells maintained in the base media. However, as the results of the immunofluorescence assay evinced, SUM-149sh4 and shPld knockdowns had similar amounts of membrane EGFR to SUM-149 and SUM-149shNS
cells. These results suggest two possibilities: either the gene knockdown was wearing off and AREG was again becoming active in SUM-149sh4 and shPld cells, or that EGFR membrane accumulation and AREG expression are independent of each other.

If the latter is true, it would then seem that stimulation with AREG maintained already-existing EGFR plasma membrane conglomerations, while stimulation with EGF broke up these structures. From further observation of the immunofluorescence assays, it seems possible that in maintaining EGFR membrane structures AREG expression causes cells to stick together in sheets, while in the knockdown cells devoid of AREG expression the cells were not nearly as confluent. This observation may shed light on a mechanism of invasive breast cancers like SUM-149- as invasive cancers migrate in sheets of cells, the expression of AREG and its consequential maintenance of the sheet of cells and the EGFR membrane structures may be essential to a cancer’s evolution from a migratory to an invasive stage. These are some of the questions that remain unanswered, but could lead to AREG being used as a potential biomarker, allowing for specific chemotherapeutic strategies.

Thus, in order to test these new conjectures about AREG expression and EGFR membrane structures in the future, I'd generate new clonal lines of SUM-149sh4 AREG knockdown cells, to ensure effective AREG knockdown, then grow these cells in different concentrations of EGF and compare the results to the results of this experiment. Western Blots highlighting the expression of proteins like e-cadherin and Rho-C (proteins that attach other proteins to membranes) in the SUM-149 and the knock-down cells would also be involved, in order to see whether cells with AREG expression attach EGFR to their membranes differently than AREG knockdown cells. Finally, I'd perform assays that demonstrate a cell line’s ability to migrate or invade, such as the Scratch Wound assay, in order to determine whether AREG expression increases the cell line’s capacity to invade.

In conclusion, although a cure for cancer has not yet been found, it is my sincere hope that each report, each assay, and each experiment is bringing us closer to that lofty goal.

References


Minocycline: A Novel Treatment to Improve Cognitive Outcome Following Traumatic Brain Injury

Uma Jasty
Traumatic brain injury (TBI) accounts for approximately one third of all injury related deaths in the United States. The Center for Disease Control reports that each year 1.7 million people endure traumatic brain injuries; of these injuries, over 50,000 result in death, while 275,000 result in hospitalization\textsuperscript{17}. In this day and age, as soldiers return from the war on terrorism, the devastating effects of TBI are becoming more apparent. New technology has led to deadly weapons such as the improvised explosive device (IED) that increase the chances of soldiers receiving a TBI\textsuperscript{19}. According to the Brain Trauma Foundation, data has shown that the rate of wounded soldiers sustaining TBI is at 33 percent, and anywhere from 150,000 to 300,000 Iraq veterans have some level of TBI\textsuperscript{2}.

Common consequences of TBI include diffuse axonal injury, cerebral ischemia, edema, focal contusions and swelling, all of which can ultimately lead to permanent cognitive and physical disabilities\textsuperscript{13}. Therefore, various organizations such as the TBI-CT Network are performing clinical studies in an effort to ameliorate the after effects of TBI in patients\textsuperscript{18}. Many clinical trials simply observe the outcomes of TBI in patients and conduct intelligence tests that look at cognitive function following TBI\textsuperscript{15}. These tests help to look at how the brain is affected upon receiving TBI and also help to provide researchers with information about what parts of the brain are impacted. Similar cognitive tests, known as behavioral tests, are carried out using animals, namely rats, which subject rats to various tests such as an open field, elevated maze, and forced swimming\textsuperscript{15}.

In addition to performing behavioral and cognitive tests, researchers have currently been working to find ways to decrease the after effects of TBI and improve cognitive function after TBI. One study investigates the effects of Endothelin-1 exertion of vasoconstrictor and vasodilator on its receptors A (ETrA) and B (ETrB)\textsuperscript{5}. This study tested the outcomes of ETrA and ETrB antagonists with induced TBI in rats and found that ETrA blockade increased blood flow to the brain after the induced TBI\textsuperscript{7}. Furthermore, rats which had received a TBI and were given BQ-123, a selective endothelin receptor A antagonist, were compared to rats with TBI who had been given BQ-788, a selective endothelin receptor B antagonist\textsuperscript{14}. Rats which received BQ-123 had significant improvement in their cognitive scores when tested in a radial maze, as opposed to rats which had received BQ-788\textsuperscript{14}.

One prominent, yet often overlooked, issue occurring in TBI is that of microglia activation in the brain after TBI. By definition, microglia are “tissue macrophages that populate the mammalian central nervous system…and by adulthood are found in all regions of the brain and spinal cord and comprise 10-15% of the total cells in the CNS”\textsuperscript{10}. Microglial cells become activated after injury or infection in order to help repair the damaged processes in the brain\textsuperscript{9}. Additionally, microglia are the main effectors in inflammatory response in the CNS\textsuperscript{12}. This inflammatory response does not always lead to a positive outcome because microglial activation targets neuro-degeneration\textsuperscript{12}. For instance, in TBI cases where brain cells are being destroyed, microglial cells do not necessarily discriminate in their ability to phagocytize cells by releasing proinflammatory and neurotoxic factors\textsuperscript{12}. This provides a rationale for inhibiting microglial activation after traumatic brain injuries.
While it is known that microglial activation can lead to such devastating outcomes, few researchers look at ways to decrease microglial activation after TBI. Minocycline is a drug known to act as an anti-inflammatory agent and provide neuro-protective properties in neurological injuries and diseases. Minocycline has also been shown to decrease microglial activation by over fifty percent following TBI. To this end, this work sought first to characterize the extent of microglial activation occurring in the brain after induced TBI in rats and secondly to test the efficacy of Minocycline on microglial activation and behavioral outcome following TBI. This study proves important because this work could ultimately save lives and prevent permanent disabilities in individuals who have suffered traumatic brain injuries.

Methods:

Animals used:

Adult (400-450 g) male Sprague-Dawley rats were purchased from Charles River Laboratories. Animal care and surgical procedures were approved and in compliance with regulations mandated by the Wayne State University School of Medicine Animal Investigation Committee following the Guide for the Care and Use of Laboratory Animals. The animal care facility is IACUC approved and in compliance with the regulations and standards dictated by the National Institutes of Health.

Closed head injury:

In order to test the effects of an injury to the brain, TBI was induced in adult male Sprague-Dawley rats by using the Marmarou impact acceleration model. Rats (n=12, 4 per group for histology; n=30, 10 per group for behavior) were initially anesthetized with 5% halothane in 2% oxygen and then maintained with 1.5% halothane throughout the duration of the experiment. Animals were placed on a Deltaphase warming pad, maintained at 37°C and rectal temperature was monitored before and after trauma. A midsagittal scalp incision was performed and the underlying muscles were retracted laterally in order to attach a 10 mm-diameter x 3-mm-thick round stainless steel helmet with cranioplastic cement to the skull. The attached helmet covered the sagittal suture and extended from bregma to lambda sutures. The helmet evenly distributed the applied force over the surface of the parietal bones, preventing skull fractures. Anesthetized animals were placed lying face down on a thick (12 cm) foam pad to allow for head acceleration after impact. A 450 g weight contained in a hollow Plexiglas cylinder- with drilled 0.5mm holes in its wall to allow for air to escape during weight drop- was first aligned perpendicularly and then dropped directly onto the helmet from a height of 2 meters. Sensory cutaneous and proprioceptive stimuli to fore and hind limbs were provided at 2 minute intervals to observe the effects of trauma on evoked motor responses. Sham-operated, control animals (n=12) were surgically treated exactly as injured animals (n=36) except for administration of weight drop impact. Minocycline animals (n=12) received injections of Minocycline, 0.1 mg per kg, following the induced TBI. Minocycline following induced TBI, Sham-control, and impacted animals were treated at the same time and in the same room in order to control for possible stress effects induced by the smell of blood, by sounds, or by other aspects involved in the technical application of the protocol.
Assessment of microglial activation:

Microglial activation in the brain was assessed by staining fixed brain sections with HRP-conjugated Isolectin B4 (ILB4). At the time of sacrifice, rats are deeply anesthetized and perfused with paraformaldehyde. Brains are then removed and stored overnight in fixative at 4°C. Sections of 50 µm thickness will be cut through the smCx and Hipp (-1.8 through – 3.8 mm with respect to Bregma) using a cryostat. The tissue sections were floated in solutions of PBS and .3% Hydrogen Peroxide. The hydrogen peroxide blocks endogenous peroxidase activity and so prevents any background staining from occurring16. Next the tissue was incubated in PBS and .1% Triton X and then incubated in PBS/.1% Triton X and ILB4 at 4°C Celsius overnight. Triton X is a detergent which is used to increase the permeability of the membrane in the cell and thus, allows the antibodies directed at ILB4 to get through the membrane and bind to the antigen, ILB4 found on the microglial cells. A final key step in the staining process is incubating the tissue in a solution of PBS and .1% diaminobenzidine (DAB) because in the reaction with Peroxidase and hydrogen peroxide, oxygen and the free radical HO form16. When the DAB is exposed to the HO, the DAB precipitates out a brown-black solution which becomes a solid and fills up the space within the microglial cells which in turn allows us to view the microglial activation under the microscope16. Through this method we are able to clearly see the different effects of induced TBI as well as the effects of Minocycline on the brain.

Microglial reactivity is viewed under the light microscope and the number of stained cells observed after various treatments will be quantified using NIH Image. Cell counts are sampled from a 1 mm² area of the smCx and Hipp by a reviewer blinded to the treatment conditions.

Behavioral testing and radial arm maze setup:

A custom designed radial arm maze was built using black acrylic sheet (0.6 cm thick). Eight identical radial arms are fixed to a central octagonal platform and are spaced equidistant around it. Each radial arm is 60 cm in length and 10 cm in width with 20 cm – high sidewalls along each arm. A white paper triangle (7 cm sides) is placed 10 cm above the base of radial arm #1. Similarly, a white paper figures of a square enclosing a circle (7 cm/side square, 3 cm circle diameter), a rectangle (5 cm x 7 cm), and a circle (7cm diameter) are placed 10 cm above the base of radial arm #3, #5, and #8 respectively. These visual cues are aimed to provide spatial guidance as to the location of the baited arm (i.e. containing the food).

The rats were allowed to acclimate to their new environment (in DLAR facility) after their arrival. Light-dark cycles were artificially instituted in 12 hour segments which were in coordination to exterior environmental conditions. Behavioral studies were consistently undertaken after the onset of the night cycle for 20 consecutive days.

The rats were tested for the time taken to find the bait (1 loop of Fruit Loop cereal®) placed 5 cm from the end of four different radial arms. Each animal has its own unique setup in the maze to ensure each rat produced its own, independent results. Also the number of spatial learning (entering an unbaited arm) and memory-retention (re-entering a baited arm after the food has been removed) errors were recorded. The rat was allowed to spend a maximum of ten minutes in the maze per trial and the end of this time period was determined to be the conclusion of a trial. Averages of two daily trials per animal were calculated and recorded.
Results:

Our results indicate that Minocycline is effective in mitigating both histological and behavioral deficits following TBI. We found that the animals injected with 1.0 mg of Minocycline per kilogram of weight had fewer activated microglial cells than TBI animals that did not receive Minocycline. Further, the animals given Minocycline performed significantly better in the radial arm maze than the TBI only animals in terms of latency.

The animals who received induced TBI had an average of 6.125 activated microglial cells with an error of ± 1.25 activated microglial cells per section (which measured 100 micrometers) in each animal. As opposed to the TBI only animals, the sham animals had an average of only 1 ± 0.45 activated microglia cell per section per animal. The number of activated microglial cells per section per animal in TBI animals injected with Minocycline fell in between that of the TBI only animals and sham animals. The rats injected with Minocycline averaged 2.875 ± 1.5 activated microglial cells in each section.

In the radial arm maze, sham animals outperformed the other groups in terms of latency, the time it took the animal to find and eat all pieces of food. Sham animals had a latency of ten minutes on the first day, but by day five the sham animals had reduced their latency to about five minutes. Sham animals continued to decrease their latency, though not as drastically as in the first five days, from days seven to eight where the latency dropped to under four minutes. Sham animals also displayed a steady, curve shaped decrease in latency from days ten to thirteen where the latency fell to just over one minute and remained at that value for the remainder of the study. The TBI only animals, however, had a latency of ten minutes from day one until day nine and displayed no significant decrease in latency. After day nine until day twelve, the TBI only animals had a steady decline in their latency which fell to around six minutes. For the remainder of the study after day twelve, the TBI only animals had a slight decrease in latency and the final average latency for TBI only animals fell to around five minutes. The TBI only animals cut their latency by 50%, as opposed to the sham animals which cut their latency by almost 90%. For the TBI animals given Minocycline, their initial latency was ten minutes just like that of the other groups. The TBI plus Minocycline animals displayed a consistent decline in latency until day fourteen at which the latency was six minutes. From day fourteen to eighteen there was a drastic decline in latency to about two minutes for TBI animals injected with Minocycline. Additionally, the TBI animals given Minocycline finished the study with a latency of just over two minutes—much lower than that of the TBI only animals, and just slightly higher than that of the sham animals.

Histological analysis and behavioral assessments ensured that the results were reliable and not skewed in any way. All histological analyses are conducted using 4 to 6 sections per animal with 3 to 6 areas of analysis per section. Data was expressed as an average of each area of analysis. In between group analysis is accomplished using one-way analysis of variance (ANOVA), with least significant difference post-hoc testing. Data were reported as mean ± SE. Significance is set at p-value < 0.05. Based on variability in these data from our previous studies, we can distinguish a difference in number of activated microglia with 95% power at an α level of 0.05 with 4–6 rats per group. In drawing comparisons with combined treatments we used a multivariate ANOVA (MANOVA) to compare across treatments so as to reduce the risk of Type I errors.
All behavioral data are expressed as the average latency of completing the task over three trials. Inter-group analyses are accomplished using one-way analysis of variance (ANOVA) with least significant difference (LSD) post-hoc testing. Data are reported as mean ± SE. Significance is set at p-value < 0.05. Due to the variability in behavior amongst individual animals, we have previously determined that we can distinguish significant differences between TBI and control animals using 10 animals per group to show an improved performance with power of 90%\(^6,8\). This allows us to distinguish a difference in latency of 2 min with 90% power at \(\alpha = 0.05\). In drawing comparisons with combined treatments we use a multivariate ANOVA (MANOVA) to compare across treatments so as to reduce the risk of Type I errors.

The results of the study answered the initial question posed at the start of the research: will the injection of Minocycline in animals with induced TBI decrease microglial activation in the brain and improve cognition in the maze? The data conclusively showed that microglial activation decreases significantly in TBI patients after the injection of Minocycline. The results displayed the differences in average microglial activation in the three groups tested: sham (control), TBI only and TBI injected with Minocycline. The data also showed that latency for TBI + Minocycline animals was markedly better than that of the TBI only animals. By demonstrating lower latency, the results of the TBI plus Minocycline animals supported the hypothesis while at the same time answering the question regarding improved cognitive functioning in TBI animals given Minocycline versus TBI only animals.
Illustrations:
Figure 1. The effects of Minocycline treatment on TBI-induced microglial activation.

Top: Sham operation was performed in an animal. 24 hrs later the brain was removed and sectioned at the level of the hippocampus. Tissue was stained for ILB4, a marker for microglial activation. A few processes were labeled, however no microglial cell bodies were detected.

Middle: TBI was induced using Marmarou’s weight-accleration impact model. Brains were removed 24 hrs later and sectioned at hippocampus level. Tissue was stained for ILB4. Results indicate that TBI results in a substantial increase in microglial activation as can be seen by the intensely stained microglial cells.

Bottom: Minocycline treatment (1.0mg/kg) dampened the microglial staining, suggesting that this dose inhibits microglial activation.
Graph 1. The effects of Minocycline treatment on TBI-induced microglial activation. Quantitative analysis of microglial activation, showing that minocycline significantly inhibits activation. *=significant from sham (p<0.005), &-=significant from TBI (p<0.005).

Figure 2. The effects of 1.0mg/kg IV injection of minocycline administered 20 min after TBI on cognitive performance in a radial arm maze. TBI and sham groups are identical to figure 4. Another set of animals was given TBI followed by a single 1.0mg/kg IV injection of minocycline which blocks microglial activation. Results indicate that, similar to ETrA antagonism, minocycline was able to improve #, *=significant (P<0.005) as compared with sham operated animals; **=significant (P<0.005) as compared with TBI.


Discussion:

Ultimately, the results of the research show that Minocycline can dramatically improve the quality of patients’ lives following TBI. In any individual, there is a small amount of activated microglial cells as shown in the Sham animals. However, in contrast, TBI only animals had a much larger number of activated microglial cells. TBI only animals had more activated microglial cells because following any brain injury, microglial cells activate. This activation can lead to further damage in the brain as discussed in the introduction. The results showed that the Minocycline greatly decreased microglial activation in the TBI + Minocycline animals as compared to that in TBI only animals. Furthermore, the microglial activation occurring in animals injected with Minocycline was only slightly larger than that occurring in the sham animals. This tells us that if injected within twenty four hours of the brain injury, Minocycline can act as an anti inflammatory in the brain and stop further microglial activation from occurring in the brain. Thus, because Minocycline proves so effective in combating microglial activation and decreasing inflammation following TBI, it lessens the degree of damage that the activated microglial cells can cause.

Additionally, Minocycline’s ability to decrease microglial cell activation shows a relationship between cognitive functioning and microglial activation. The more activated microglial cells, the worse the cognitive functioning, as the results of the radial arm maze tests showed. The TBI only animals did not cut their average latency in half by the end of the study. On the other hand, the Minocycline animals decreased their latency by more than half by day twenty. At the end of the study, the Minocycline animals were performing almost as well as the sham animals. The Minocycline animals demonstrated a better aptitude for memorizing the maze than the TBI only animals. The sham animals had the least number of activated microglial cells and performed the best in the maze. Not only that, but latency measures the time it takes the animal to find and eat all four pellets of food. So, the better the latency, the better the animals’ ability to recollect information. Therefore, decreasing number of activated microglial cells in the brain not only prevents further injury from occurring, but also clearly leads to better cognitive function following TBI.

It is important to note that Minocycline is a drug that is readily available to all individuals and is inexpensive as opposed to other prescription drugs. For that reason, Minocycline could be immediately utilized by any individual suffering from TBI to prevent further damage from occurring in the brain tissue and to help increase cognition and memory retention after TBI. More specifically, Minocycline could be used for current soldiers in Iraq and Afghanistan who receive TBI from implosive explosive devices and other weapons: by quickly injecting any soldier receiving TBI with Minocycline, soldiers could be given the chance to recover to their original cognitive functioning.

The observed results in this work are supported by the studies conducted by Abdel, Schwab and others regarding the effects of Minocycline combined with other drugs on rats induced with TBI. Abdel’s study found that together Minocycline and N-acetylcysteine do in fact improve cognition and memory after TBI. Thus, the results of this research not only confirm their observations, but also suggest that, perhaps, combined treatments are not necessary, thus decreasing the cost to those already burdened by the effects of TBI.
Other published articles that research TBI look at similar methods and drugs that can be used to improve cognition after TBI. One study found Minocycline had beneficial effects on cerebral edema following TBI. This study discovered that Minocycline proved effective in reducing cerebral edema and also in reducing inflammatory markers\(^3\). In another similar study, Minocycline was shown to reduce TBI induced motor hyperactivity- manifestation of mania or depression following TBI. This same study also stated that the short term effects of Minocycline on microglial activation led to long lasting functional outcomes\(^4\). Thus, these studies went hand in hand with this research and showed the consistency in the findings set forth in this paper. All these studies demonstrated the positive effects of Minocycline on the brain following TBI. The two studies also showed the anti-inflammatory effects of Minocycline on the brain and the findings explained in this report further enhance these results by including the results of a radial arm maze test. The results of the radial arm maze provide further evidence that Minocycline has positive effects on the brain and leads to improve functioning post-TBI. At the same time, however, this study proved unique because it studied Minocycline’s effect on microglial activation- not cerebral edema or hyperactivity, the subjects of previous studies.

This research, as well as other research, looks at the significance of Minocycline on TBI patients. TBI is an injury resulting in devastating outcomes that Minocycline can help to improve through reduction of microglial activation after TBI and thus, improvement of cognition.

**Conclusion:**

Through this research, it was in fact found that Minocycline proves effective in decreasing the amount of microglial activation in post-TBI animals, as depicted in the pictures of the stained microglial cells. The results of the radial arm maze research verified that Minocycline also successfully improves cognitive functioning in animals. Thus, future work will be designed to further test the mechanism by which inhibiting activated microglia may be linked to improved cognition, ultimately leading to potential clinical trial using Minocycline as a novel treatment strategy to improve outcome in those suffering the effects of TBI.

The conclusions drawn from this research stem from both the report and from prior studies on TBI. These results are supported and also go hand in hand with other research reports like those mentioned in the discussion. Thus, the results of the research proved valid because they not only offered conclusive findings, but also because other studies have come to similar conclusions on the effectiveness of Minocycline on TBI patients.

In the future, I hope to refine my results by conducting experiments that also look at the side effects of Minocycline. Side effects like nausea may have contributed to weak performances by the TBI + Minocycline animals at the start of the study. Furthermore, I would conduct the same experiment, but I would use a larger sample size of about 12 animals in each group to further validate the results that Minocycline does in fact decrease microglial activation in all TBI.

Had I more time, I would have liked to conduct the radial arm maze studies for a period of six weeks, not for just twenty days. By studying the animals’ behavior in the maze for a longer period of time, I believe I would be able to better observe whether Minocycline proves more effective immediately following the injection or after a prolonged time. Also, I would have liked
to look at Minocycline’s affect on activated microglial cells after six weeks because in my study I only looked at Minocycline’s immediate effects on the brain after one day. If I were to start from scratch I would add different experiments. Firstly, I would like to look at the role of an enriched environment on cognition. Other studies have shown that there appears to be a connection between a stimulated environment and improved cognitive functioning. Therefore, my goal would be to look to see if a combined effort of Minocycline and an enriched environment led to a better cognitive outcome than just Minocycline. Many questions about TBI remain unanswered. Studying the brain proves difficult because it is such a complex yet vital organ. However, the ultimate question that remains for traumatic injury studies is whether any drug will ever be able to reverse the effects of TBI, or more realistically whether any drug will be able to return cognitive functioning of TBI patients back to the original level of functioning.

References


Alcohol Consumption During Pregnancy and Facial Dysmorphismology in Offspring at 6-7 Years of Age

Natasha Sood
Fetal Alcohol Spectrum Disorder (FASD) is a collection of permanent birth defects that result from maternal alcohol ingestion during pregnancy. Children with FASD may have mental and behavioral impairments, as well as facial and growth abnormalities with lifelong implications. FASD occurs in approximately 9 per 1000 births and is the leading preventable cause of mental retardation in the Western hemisphere.

The disabilities associated with FASD are compounded by secondary emotional and behavioral consequences such as low self-esteem, depression, school failure, and criminality during later childhood and adult life. These secondary disabilities come at a high cost to the individual, his or her family, and society and may be reduced by early diagnosis and appropriate intervention.

Problem

Identification of FASD is challenging as there is no confirmatory laboratory test. The diagnosis rests primarily on history of maternal drinking which may not be forthcoming at presentation. Physical examination characteristics and behavioral symptoms may be subtle or manifest later in life and change over time. The most specific feature of FASD is the facial phenotype. Therefore, without reliable information regarding maternal use of alcohol during pregnancy or maternal neurobehavioral problems, the majority of health care providers have to rely on characteristic facial features of FASD such as hypoplastic philtrum, thin upper lip, short nose relative to the midface length, short palpebral fissures, and a flattened maxillary region.

Although the facial features are a key diagnostic component of the syndrome, they are minor anomalies, which are usually of little medical consequence to the individual. Of greater significance is the fact that the facial anomalies are midline anomalies derived from the anterior frontal neural crest primordia of the early forebrain. It has long been speculated that some midline facial anomalies are pathognomonic of brain malformation (i.e., the face predicts the brain) and therefore the existence of FAS facial features may suggest significant brain and nervous system damage. The critical period for the induction of FAS-like craniofacial malformations occurs very early in gestation and is very short in duration.

There are no accurate, precise and unbiased methods for measuring and recording facial dysmorphology. Hence, even children with the full spectrum of FASD, including facial dysmorphology, may go undetected both in the newborn period and even later in life. The FASD facial dysmorphology is known to change as the child ages but is most recognizable around the age of five. There may also be population or ethnic differences in the expression of the characteristic facial features associated with the syndrome.

To standardize the evaluation of FASD, Astley and Clarren developed a 4-Digit Diagnostic Code based on objective measurement scales, for diagnosing the full spectrum of abnormalities among FASD patients. The four digits in the code reflect the magnitude of expression of the four key diagnostic features of FASD in the following order: (1) growth deficiency; (2) the FAS facial phenotype assessed on standardized digital photographs; (3) central nervous system damage/dysfunction; (4) gestational alcohol exposure. The magnitude of expression of each feature is ranked independently on a four-point Likert scale with 1 reflecting
complete absence of the FAS feature and 4 reflecting a strong ‘classic’ presence of the FASD feature.

The three key diagnostic facial features used to characterize the FASD facial phenotype in the 4-Digit Diagnostic Code are: the small palpebral fissures, a smooth philtrum, and a thin upper lip. Palpebral fissure length (PFL) $z$-scores are computed with adjustment for age and when possible race. The thinness of the vermillion border of the upper lip and the smoothness of the philtrum are coded independently on a 5-point pictorial Likert scales. The magnitude of each of these facial features is ranked by an ABC score, which is then converted to the 4-Digit Diagnostic Code rank for the face using a software program$^{2,4}$.

The purpose of this study was to objectively measure the facial features of African-American children born to women with known alcohol consumption status during pregnancy, and to correlate the abnormalities in the facial features to the amount of maternal alcohol consumption during pregnancy. In addition, prenatal alcohol exposure (PAE) status was correlated with birth weight, length, head circumference and gestational age of the patient. The hypothesis was that facial morphogenic features, birth growth parameters and gestational age of children born to women who use alcohol during pregnancy is associated with amount and timing of alcohol ingested and may be affected by the gender of the offspring such that different effects may be seen in male and female offspring.

Specific Aims
1) To objectively measure and compare the facial features of 6-7 year-old children born to women who consumed alcohol during pregnancy to the children of women who did not.

2) Correlate the abnormalities in the facial features to the amount of maternal alcohol consumption during pregnancy.

3) Determine whether prenatal alcohol exposure (PAE) correlates with birth weight, length, head circumference and gestational age of the patient and to determine if gender moderates the effects of prenatal alcohol exposure on growth parameters at birth and facial features at 6-7 years of age.

Materials

Procedure
Subjects:
Participants of this study were offspring born to African American women who received prenatal care at Wayne State University between 1988-1991. These women were screened during pregnancy for use of alcohol - frequency and the type of alcohol used. Pre-pregnancy and
current alcohol intake was elicited to determine day-by-day alcohol intake for the periconceptional period and the 2 weeks preceding the visit. Women who abstained from alcohol use formed a control sample to identify the associations between alcohol intake and child outcomes. Over 90% of women seeking prenatal care at this site were African-American. Therefore, because of inadequate representation of other racial groups, data were collected only from these African-American women. These families have been followed since then and the offspring evaluated for various outcomes at different ages. For this study, standardized, de-identified photographs meeting certain minimum criteria of 6-7 year old African American children born to these women were analyzed for FASD facial phenotype. In addition information regarding growth parameters at birth, gestational age and gender were available as part of the research being conducted at Wayne State University. This information was made available as a de-identified data set for this study after approval by the Wayne State University Institutional Review Board.

**Independent Variable – Prenatal Alcohol Exposure:**

The independent variable in this study, prenatal alcohol exposure (PAE), was computed as the average absolute alcohol per day across pregnancy. At each prenatal visit, mothers were interviewed about alcohol use during the previous 2 weeks. Quantities and types of alcohol consumed were converted to fluid ounces of absolute alcohol and averaged across visits to generate a summary measure of alcohol exposure throughout pregnancy as the \textit{average ounces of absolute alcohol per day} (AAD). To evaluate the effects of different levels of exposure, the average absolute alcohol intake was relatively arbitrarily categorized into none, low(>0 but<0.75 fl oz of absolute alcohol/day), and moderate-heavy (\geq 0.75 fl oz of absolute alcohol/day) for the purpose of this study. There is no uniformly accepted definition of “low”, “moderate” or “heavy” alcohol use during pregnancy in the literature.

**Outcome Variable – FASD Facial Phenotype Determined by Analysis of Standardized Digital Photographs:**

Standardized digital photographs were evaluated for three key diagnostic features that characterize the FAS facial phenotype: small palpebral fissures, smooth philtrum, and thin upper lip using the computerized FAS Facial Photographic Analysis Software. Formal online training course on the use of the FAS Facial Photographic Analysis Software and the 4-Digit Diagnostic Code was undertaken by the author of this paper.

**Palpebral Fissure Length:**

To determine the palpebral fissure length (PFL), a digital photo of the face taken with a paper sticker placed between the eyebrows to serve as an internal measure of scale, was analyzed using the FAS Facial Photographic Analysis Software. The distance between the endocanthion (en) and the exocanthion (ex) was measured in mm for the right and left eye (Figure 1).
The z-score for PFL was calculated as the number of standard deviations above or below the norm of the mean patient’s PFL:

$$z-score = \frac{(patient's\ PFL - mean\ PFL\ for\ normal\ population)}{(Standard\ deviation\ of\ mean\ PFL\ for\ normal\ population)}$$

Normal PFL charts for Caucasian children were used to compute the z-score. Normal PFL charts adjusted for race are not available. The PFL was assigned a ABC rank according to its z-score.

The distance between the right endocanthion and the left endocanthion was also measured - this is called the Inner-canthal distance.

**Upper Lip Thinness and Flat Philtrum:**

The upper lip thinness and philtrum smoothness were measured using the 5-point pictorial Likert scale, using the Lip Philtrum Guide. In order to obtain accurate measures, the lips must be gently closed with no smile. Separate guides are available for Caucasians and African Americans – in this study the guide for African Americans was used (Figure 2).

Next the upper lip thinness was also calculated by outlining the upper lip with the computer mouse to compute the circularity ($perimeter^2/area$) as shown below in Figure 3.

As the upper lip becomes thinner, the circularity increases. Circularity is not influenced by the size of the photograph. The software automatically ranks the lip thinness based on the circularity. This rank is independent of the lip thinness rank assigned using the 5-point Likert rank for the shape of the upper lip on the Lip Philtrum Guide.
**Deriving the Facial ABC-Score:**
The PFL, philtrum smoothness and upper lip thinness were ranked using the ABC score table (Table 1).

<table>
<thead>
<tr>
<th>5-Point Likert Rank for Philtrum and Lip</th>
<th>Z-score for Palpebral Fissure Length</th>
<th>Circle the ABC-Scores for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 or 5</td>
<td>≤ -2 SD</td>
<td>C</td>
</tr>
<tr>
<td>3</td>
<td>&gt;-2 SD and ≤ -1 SD</td>
<td>B</td>
</tr>
<tr>
<td>1 or 2</td>
<td>&gt;-1 SD</td>
<td>A</td>
</tr>
</tbody>
</table>

**Table 1: Deriving the ABC-Score for Facial Phenotype (Astley 2004)**

**Deriving the 4-Digit Rank for Face:**
The individual ABC scores (which are derived from the Likert scales) were combined by the software to generate an overall 4-Digit Diagnostic Rank (Table 2).

<table>
<thead>
<tr>
<th>4-Digit Diagnostic Rank</th>
<th>Level of Expression of Facial Features</th>
<th>Palpebral Fissure – Philtrum – Lip ABC-Score Combinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Severe</td>
<td>CCC</td>
</tr>
<tr>
<td>3</td>
<td>Moderate</td>
<td>CCB, CBC, BCC</td>
</tr>
<tr>
<td>2</td>
<td>Mild</td>
<td>CCA, CAC, CBB, CBA, CAB, CAA, BCB, BCA, BBC, BAC, ACC, ACB, ACA, ABC, AAC</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>BBB, BBA, BAB, BAA, ABB, ABA, AAB, AAA</td>
</tr>
</tbody>
</table>

**Table 2: Converting the Facial ABC-Score to a 4-Digit Diagnostic Rank for Face (Astley 2004)**

**Statistical Analysis:**
Statistical analyses were done using the SPSS statistical package. Frequency distribution, cross tabulation and chi-square tests were used to evaluate the relationship between categorical AAD variable and categorical outcomes like ABC scores for PFL, philtrum, the upper lip thinness and the 4-Digit Diagnostic Code. Analysis Of Variance (ANOVA) was used to evaluate the relationship between the three category AAD variable and continuous outcomes like birth weight, birth length, head circumference, gestational age and the PFL z-score. Ordered logistic regression analysis in SPSS was performed to determine if PAE was a predictor for facial phenotype after controlling for confounding variables including gender, birth weight, gestational age at birth, and age at obtaining standardized photograph. The sensitivity and specificity of the PFL ABC rank and the 4-Digit Rank for Face in predicting any PAE was also calculated.
Data

Sample Characteristics:

Of the 506 parent-child dyads in the parent study, standardized digital photographs were available for 95 subjects at 6-7 years. Of these, 67 had no, 16 had low and 12 had moderate-severe prenatal alcohol exposure (PAE). The mean ± SD age of the subjects at the time of obtaining standardized photographs was 6.9 ± 0.3 years and 51 (53.7%) were male. The mean age and gender distribution did not vary by prenatal alcohol exposure group.

The offspring of mothers who used alcohol during pregnancy had a lower birth weight compared offspring of mothers with no PAE (Table 3, Figure 4). The mean birth weight of infants with no PAE was 3.2 Kg. Infants with either low or moderate-severe PAE were smaller (birth weight, 2.6 and 2.6 kg respectively, p=0.001). There was no difference in birth weight of infants with low compared to infants with moderate-severe alcohol exposure groups.

The mean birth length of infants with no, low, and moderate-severe PAE was 49.6, 45.6, 45.2 cm respectively (p=0.000).

The mean head circumference at birth of infants with no, low, and moderate-severe PAE was 33.9, 31.4, 32.0 cm respectively (p=0.000). This pattern was similar to that for birth weight. Infants with low or moderate-severe alcohol exposure were born at a significantly earlier gestational age (36.9, 36.8 weeks respectively) as compared to infants with no PAE (38.6 weeks; p=0.009).

Table 3: Characteristics of Study Subjects:

<table>
<thead>
<tr>
<th>Prenatal Alcohol Exposure Group</th>
<th>None (N=67)</th>
<th>Low (N=16)</th>
<th>Moderate-Severe (N=12)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth Length</td>
<td>49.6±3.1</td>
<td>45.6±5.0</td>
<td>45.2±4.5</td>
<td>.000</td>
</tr>
<tr>
<td>Birth Weight</td>
<td>3.2±0.6</td>
<td>2.6±0.8</td>
<td>2.6±0.8</td>
<td>.001</td>
</tr>
<tr>
<td>Birth Head Circumference</td>
<td>33.9±1.8</td>
<td>31.4±3.2</td>
<td>32.0±3.3</td>
<td>.000</td>
</tr>
<tr>
<td>Birth Gestational Age</td>
<td>38.6±1.8</td>
<td>36.9±3.2</td>
<td>36.8±4.0</td>
<td>.009</td>
</tr>
<tr>
<td>Male Gender - n (%)</td>
<td>39 (58)</td>
<td>6 (38)</td>
<td>6 (50)</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years) - mean ± SD at obtaining photograph</td>
<td>7.0 ± 0.3</td>
<td>6.9 ± 0.3</td>
<td>6.9 ± 0.2</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 4: Relationship of Categorical Prenatal Alcohol Exposure and Characteristics at Birth:

Orange squares represent mean and maroon bars represent 95% confidence limits of the mean
**Relationship of Prenatal Alcohol Exposure to Facial Phenotype at 6-7 years of age:**

The mean palpebral fissure length (PFL) measured in millimeters was higher and the PFL z-score was significantly lower in infants with no PAE. The mean PFL z-score in infants with no, low, moderate-severe PAE was -1.3, -1.4 and -2.3 respectively. The difference between infants with no and low PAE was not significant but these two groups were significantly different from the infants with moderate-severe PAE (p=0.002). The inner-canthal distance was not significantly different between the three groups (Table 4).

**Table 4: Palpebral Fissure Length and Inter-Canthal Distance by Prenatal Alcohol Exposure:**

<table>
<thead>
<tr>
<th>Prenatal Alcohol Exposure</th>
<th>None N=67</th>
<th>Low N=16</th>
<th>Moderate-Severe N=12</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palpebral Fissure (mm)</td>
<td>2.9±0.2</td>
<td>2.9±0.2</td>
<td>2.7±0.4</td>
<td>.003</td>
</tr>
<tr>
<td>Palpebral Fissure (z-score)</td>
<td>-1.3±0.8</td>
<td>-1.4±0.8</td>
<td>-2.3±1.2</td>
<td>.002</td>
</tr>
<tr>
<td>Inner Canthal Distance (mm)</td>
<td>3.4±0.4</td>
<td>3.3±0.3</td>
<td>3.2±0.5</td>
<td>.177</td>
</tr>
</tbody>
</table>

**Figure 5: Relationship of Categorical Prenatal Alcohol Exposure and Facial Features at 6-7 Years of Age:**

*Orange squares represent mean and maroon bars represent 95% confidence limits of the mean*
A comparison of the ABC scores for the PFL, philtrum and upper lip thinness, showed significant differences between the three groups only for PFL (p=0.004) (Table 5, Figure 6). The 4-Digit Rank for Face, which is derived from a combination of the ABC scores for PFL, philtrum and upper lip thinness, was also significantly different between the three groups (p=0.043). It seems that the 4-Digit Rank for Face is primarily determined by the ABC score for PFL and therefore PFL alone may be used for evaluating prenatally alcohol exposed children. The effects of PAE on facial phenotype were seen only with moderate-severe prenatal alcohol exposure.

Table 5: ABC Ranking of Facial Features by Prenatal Alcohol Exposure Group

<table>
<thead>
<tr>
<th>Rank</th>
<th>None</th>
<th>Low</th>
<th>Moderate-Severe</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palpebral Fissure Length</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>31%</td>
<td>19%</td>
<td>17%</td>
<td>0.004</td>
</tr>
<tr>
<td>B</td>
<td>46%</td>
<td>56%</td>
<td>8%</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>22%</td>
<td>25%</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td>Philtrum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>16%</td>
<td>19%</td>
<td>8%</td>
<td>0.66</td>
</tr>
<tr>
<td>B</td>
<td>25%</td>
<td>38%</td>
<td>42%</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>58%</td>
<td>44%</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>Lip</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1%</td>
<td>13%</td>
<td>0%</td>
<td>0.191</td>
</tr>
<tr>
<td>B</td>
<td>87%</td>
<td>81%</td>
<td>83%</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>12%</td>
<td>6%</td>
<td>17%</td>
<td></td>
</tr>
<tr>
<td>4-Digit Diagnostic Code</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>27%</td>
<td>44%</td>
<td>8%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>57%</td>
<td>38%</td>
<td>42%</td>
<td>0.043</td>
</tr>
<tr>
<td>3</td>
<td>16%</td>
<td>19%</td>
<td>50%</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6: Relationship of Categorical Prenatal Alcohol Exposure and Ranking of Facial Features at 6-7 Years of Age:

*A, B and C represent increasing severity of facial features*
**Regression Analysis:**

Ordered Logistic Regression showed that PAE was a significant predictor for palpebral fissure length ABC score (p=0.034) and the 4-Digit Diagnostic facial score (p=0.039) but not for lip thinness and smooth philtrum ABC score after controlling for confounding variables including gender, birth weight, gestational age at birth, and age at obtaining standardized photograph. Gender was not a significant moderator of facial feature abnormalities in this study.

**Sensitivity and Specificity of the Facial Phenotype in predicting PAE:**

The ABC rank for PFL had a sensitivity of 82% and a specificity of 31% in predicting any PAE as self-reported by the mother. The 4-Digit Rank for face had a sensitivity of 73% and a specificity of 27% in predicting any PAE as self-reported by the mother.

**Analysis**

This study showed that children who were exposed to prenatal alcohol had smaller growth parameters and lower gestational age at birth; and smaller palpebral fissure length (PFL) at 6-7 years. Effects on growth were observed with low PAE, whereas effects on FAS facial phenotype were evident only at moderate-severe PAE. This is likely explained by the fact that the critical period for the induction of FAS-like craniofacial features occurs very early in gestation and is very short in duration, whereas effects on growth may occur over a longer period during pregnancy. Gender was not found to moderate the facial phenotype effects of PAE.

Among the three facial features included in the 4-Digit Diagnostic Rank for face, only PFL was significantly associated with PAE; philtrum smoothness and upper lip thinness were not associated with PAE. The 4-Digit Rank for face was significantly associated with PAE, but it seems that the 4-Digit Rank for face is primarily determined by the ABC score for PFL and therefore PFL alone may be used for evaluating prenatally alcohol exposed children.

Although the PFL ABC score has a good sensitivity for predicting PAE exposure status, the specificity is low. This may be related to three factors: use of Caucasian norms for PFL in this study of African American children; inaccurate reporting of PAE by mothers; and inherent characteristics of PFL. More accurate and valid norms that are race-specific are needed for facial features. Since maternal self-report of alcohol use during pregnancy may be unreliable it is important to have a biomarker to objectively measure prenatal alcohol exposure and objectively evaluate the specificity of the facial phenotype for FASD and predict outcomes in offspring.

Although the results of our study are significant, there are limitations. Despite the fact that detailed prenatal alcohol use was determined by maternal self-report at each prenatal visit, the mother could have misreported her alcohol consumption intentionally or unintentionally. Moreover, information about the timing of alcohol use during pregnancy was not available for this study. Second, there were few subjects with moderate-severe prenatal alcohol exposure (N=12). In fact using the 4-Digit Diagnostic Code for FAS Facial Phenotype, none of the subjects were classified under the severe facial phenotype. Third, although detailed information of alcohol use by the mother during pregnancy was available, data regarding other medical problems and recreational drug use was not made available and these might have affected growth parameters at birth. Lastly, the diagnosis of Fetal Alcohol Spectrum Disorders is based on the
expression of four key diagnostic features: (1) growth deficiency, (2) FAS facial phenotype, (3) Central Nervous System abnormalities, (4) prenatal alcohol exposure. In this study growth deficiency, FAS facial phenotype and PAE were evaluated; however Central Nervous System abnormalities were not evaluated. Future studies with a larger number of subjects with PAE are needed to further study effects of prenatal alcohol.

Conclusion

This study showed that children who were exposed to prenatal alcohol had smaller growth parameters, lower gestational age at birth, and smaller palpebral fissure length (PFL) at 6-7 years. Effects on growth were observed with low PAE, whereas effects on FAS facial phenotype were evident only at moderate-severe PAE. Gender was not found to be a moderator of the facial phenotype effects of PAE.

Among the three facial features included in the 4-Digit Diagnostic Rank for face, only PFL was significantly associated with PAE. The 4-Digit Diagnostic Rank for face was significantly associated with PAE, but it seems that the 4-Digit Diagnostic Rank for face is primarily determined by the ABC score for PFL and therefore PFL alone may be used for evaluating prenatally alcohol exposed children.

Although PFL ABC score has a good sensitivity for predicting PAE exposure status, the specificity is low. More accurate and valid norms that are race-specific are needed for facial features. Since maternal self-report of alcohol use during pregnancy may be unreliable it is important to have a biomarker to objectively measure prenatal alcohol exposure to objectively evaluate the specificity of the facial phenotype for FASD and predict outcomes in offspring. Future studies with larger number of subjects with prenatal alcohol exposure are needed to further study effects of prenatal alcohol.

Summary

Fetal Alcohol Spectrum Disorder (FASD) is a collection of permanent birth defects resulting from prenatal alcohol exposure (PAE) and is the leading cause of mental retardation. The diagnosis rests primarily on history of maternal drinking, which may not be forthcoming. Alternatively, it is often based on facial abnormalities in palpebral fissure length (PFL), smooth philtrum, and thin upper lip evaluated using standardized photographs as described by Astley et al. The purpose of this study was to objectively measure the facial features of 6-7 year-old African-American children born to women with known alcohol consumption during pregnancy, and to correlate these abnormalities to the amount of PAE and to childhood characteristics. This study showed that PAE was associated with smaller growth parameters, lower gestational age at birth, and smaller PFL at 6-7 years. Effects on growth were observed with low PAE, whereas effects on facial features were seen only at moderate-severe PAE. Although PFL had a good sensitivity for predicting PAE, the specificity was low. Since maternal self-report of alcohol use during pregnancy may be unreliable, it is important to have biomarkers that objectively measure PAE in order to evaluate the specificity of the facial features and predict childhood outcomes.
References


Proteasome Inhibition and Apoptosis by the Designated DSF-analogue #39 in MDA-MB-231 Breast Cancer Cells

Shirley Wu
Angiogenesis is a key process in tumor metastasis\textsuperscript{5-8}. It is known that without the process of angiogenesis, which occurs with the trace element copper, a tumor is incapable of growing larger than 1 to 2 mm\textsuperscript{3}, showing the importance of copper to tumors\textsuperscript{17}. There is increasing evidence that many types of human cancers, including breast, prostate, and brain, have tumor cells able to acquire an abundance of copper\textsuperscript{4,9-10,12,14,16,21}. In contrast, normal cells have a limited amount of copper, which is strictly regulated by the body.

Apoptosis is a conserved and controlled programmed cell death that is found in all multicellular organisms\textsuperscript{11}. Apoptosis plays a crucial role in maintaining homeostasis and development within the body by degrading pathogen-invaded cells, cells with degraded DNA, or unwanted cells. It has been shown that many tumors have apoptotic controlling genes that are suppressed, over expressed, or altered by mutations\textsuperscript{20}. By the suppression of apoptosis inducing genes or the over expression of genes that result in cyclins for the progression of the cell cycle, cancer cells are able to proliferate. Inducing apoptosis is the main goal for controlling or stopping the growth of tumors.

The ubiquitin-proteasome pathway, which degrades cellular proteins, is one of the most important strategies in regulating apoptosis. This pathway also plays a role in the cell cycle, angiogenesis, and differentiation, all key processes for the proliferation and metastasis of tumors\textsuperscript{15}. It has been found that the inhibition of the chymotrypsin-like activity, a peptidase activity in the 20S proteasome, but not other proteasomal activities such as trypsin-like or peptidylglutamyl peptide hydrolyzing-like activities, creates a strong stimulus in inducing apoptosis\textsuperscript{1,13,18}. The induction of apoptosis is created when the proteasome is inhibited. Inhibition of the proteasome results in the accumulation of ubiquitinated proteins such as caspase 3, Bax, Parp, and p-27, ultimately producing cell death. Caspase 3, an apoptosis-inducing protein, cleaves Parp, an enzyme responsible for DNA synthesis, resulting in apoptosis, because of the inability to replicate DNA and split into two cells\textsuperscript{2}. Bax and p-27, proteins that are regularly targeted for degradation, are in the apoptosis signal pathway and indirectly cause apoptosis.

In prior studies it has been demonstrated that disulfiram (DSF), a clinically used anti-alcoholism drug, is able to bind to copper forming a potent DSF- Cu complex that inhibits proteasome activity, inducing apoptosis in human breast cancer cells \textit{in vitro} and inhibiting human breast cancer xenograft growth \textit{in vivo} without obvious toxicity\textsuperscript{3}. DSF is considered a restraint in alcohol abuse as well as cocaine abuse. When DSF comes into contact with ethanol, a DSF-Ethanol reaction (DER) occurs. DSF blocks the enzyme aldehyde dehydrogenase (ALDH), consequently irreversibly inhibiting acetaldehyde oxidation, and an accumulation of acetaldehyde results. The acetaldehyde build-up results in a discomfort that produces a flushed face, and prevents alcoholics from drinking more alcohol\textsuperscript{19}. However, the DER can have fatal effects, and DSF has vast toxicity in many areas of the body. DSF by itself, with or without ethanol, can inhibit many other drug metabolism enzymes, along with toxic metabolite formation, producing acute and chronic toxicities to the liver, the brain, and other organs\textsuperscript{19}.

Based on the literature review and previous data, the hypothesis was made that DSF-analogues would maintain anti-proliferative effects in cancer cells, while having less toxicity to normal cells due to the chemical modifications.
Materials

CuCl2, DSF (DSF), 3-[4,5-dimethyltiazol-2-yl]-2.5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). DMEM/F12, fetal bovine serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). A variety of DSF analogues were kindly provided by collaborating chemists in China.

Methods

Cell culture and lysates preparation

MDA/ MB 231 breast cancer cells were cultured in 1:1 DMEM/ F2 media containing 10% (v/v) fetal bovine serum and 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 292 µg /ml of glutamine. MCF-10A cells were cultured as previously described. All of the cells were placed in a 37°C humidified incubator with an atmosphere of 5% CO2. Then cells were harvested, washed with PBS twice, and homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP40 (v/v), 0.5 mM phenylmethylsulfonylfluoride, and 0.5 mM dithiothreitol for 20 min at 4°C. The lysates were then centrifuged at 13,000 g for 16 minutes, and the supernatants were collected as whole cell extracts.

Mixtures of Organic Copper Compounds:

The compounds were weighed and the solvent DMSO was added according to calculated values relying on their molecular weight and mass. 100 or 50 millimolar (mM) solutions of the compounds CuCl2 and DSF in DMSO were made, followed by the addition of DMSO to form 40 mM solutions. CuCl2 solutions of 40 mM were made from the 50 mM copper solution and mixed with each of the compound solutions, as well as DSF, forming 2:1 compound- CuCl2 mixtures. After, 10 mM solutions were made from DMSO and each 40 mM mixture.

Cell Viability/ Proliferation assay

The MTT assay was used to determine the effect of the various compounds on the overall proliferation of MDA-MB-231 breast cancer cells. Cells were plated in 96-well plates and grown up to 70-80% confluency, following with the addition of 10 µM concentration of the compound solutions, CuCl2 alone, compound- CuCl2 (2:1) solutions, DSF, and DMSO for 24 h. After a 24-h incubation at 37°C, the medium was removed and replaced with 100 µl of 3-[4,5-dimethyltiazol-2-yl]-2.5-diphenyltetrazolium bromide (MTT) for 2-3 h, then MTT was removed and 100 µl of DMSO was added (Added three wells with MTT and DMSO as the background). Then a colorimetric analysis was performed using a multi-label plate reader at 560 nm (Victor3; PerkinElmer (Wellesley, MA, USA)). Additional MTT assays were performed in the same condition, but using different concentration of tested DSF analogues.

Cellular morphology analysis

A Zeiss (Thornwood, NY, USA) Axiovert 25 microscope was used for all microscopic imaging with phase contrast for cellular morphology of Petri dishes of MDA-MB-231 cells treated with different concentrations of the selected compound– CuCl2 mixtures(#39, #3, & #18).

Western Blot Protocol

MDA/MB 231 Cells were seeded in 14 petri dishes and grown to 70-80% confluency, followed by the addition of selected compound #39, #3, and #18 and CuCl2 mixtures at
concentrations of 0.5 µM, 1.0 µM, and 2.0 µM. Lysates preparation was done, and whole-cell extracts were separated by SDS-PAGE gel and then transferred to a nitrocellulose membrane. The Western Blot Analysis was conducted using specific primary and secondary antibodies for β-actin, PARP, BAX, and ubiquitin. Afterwards, using the enhanced chemiluminescence Reagent, protein amounts were visible on the autoradiography film.

**Proteasomal Chymotrypsin-like activity assay**

Whole-cell extracts (10 µg protein) of MDA-MB-231 cells treated with the selected compound - CuCl₂ mixtures (#39, #3, and #18) for 16 hrs were incubated for 2 hours at 37°C in 100 µl assay buffer (10 mmol/l Tris–HCl, pH 7.5) with 10 µmol/l fluorogenic substrate Suc-LLVY-AMC (for proteasomal chymotrypsin-like activity) in the Isotemp Incubator. After incubation, production of free hydrolyzed 7-amino-4-methylcoumarin (AMC) groups liberated by substrate hydrolysis was fluorometrically measured using a Victor 3 Multilabel Counter with an excitation filter of 380 nm and an emission filter of 460 nm (PerkinElmer, Boston, MA, USA).

**Results:**

1. **Initial screening test of a variety of DSF analogues’ effect on cancer cell proliferation**

   In this experiment, mixtures of the analogs with copper chloride in a 2:1 concentration with a 1:1 volume was created. MDA-MB-231 cells, which are highly metastatic, were treated with these mixtures of a concentration of 10 µM, following with a MTT assay. Copper alone with specific concentrations and the analogues with DMSO were also used on the cells. DSF-Cu was the positive control, and DMSO was the negative control.
**Figure 1**

(A) The Cell Viability of MDA-MB-231 Cells Treated with Compound to Copper (2:1) Mixtures (10 μM)

(B) The Cell Viability of MDA-MB-231 Cells Treated with a Compound to Copper (2:1) Mixture (10 μM)

Fig. 1. The anti-proliferative effects of the DSF-analogue-copper complexes. (A) and (B) MDA-MB-231 cells were treated for 24 hours with all DSF-analogue-copper mixtures (10 μM). After 24 hrs the medium was removed and 3-[4,5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide (MTT) was added. Using a multi-label plate reader at 560 nm, cell viability of the cells was measured. DMSO acted as the negative control, and DSF-Cu the positive control. DSF-analogues #3, #7, #17, #28, #31, #39, #40, and #48 with copper had anti-proliferative effects on the breast cancer cells.

As shown in Figure 1A and 1B, compounds #3, #7, #17, #28, #31, #39, #40, and #48 with copper (Concentration of 2:1) at a concentration of 10 μM had a ~98% growth inhibition in the cultures of MDA-MB-231 cells, even more potent than the positive control (DSF-Cu). A few other compounds had ~70% inhibition of cell proliferation at a concentration of 10 μM. The remaining compounds had little or no effect on the proliferation of MDA-MB-231 cells.

Then, compounds #3, #4, #8, #13, #18, #20, #23, #25, and #39 were chosen for further tests, due to their anti-proliferative effects as shown in Figure 1A and 1B, their lesser toxicity to normal 10A cells (data not shown), as well as their solubility in DMSO.
Figure 2

(A) MTT Assay of MDA-MB-231 cells Treated with Compound-Copper Mixtures of Interest

(B) Structures of DSF-analogues made in a Chinese lab, #3, #18, #39.

(C) The Cell Viability of MDA-MB-231 Cells Treated with #3-Cu, #18-Cu, #39-Cu in Different Concentrations

Fig. 2. Inhibition of cell proliferation in MDA-MB-231 cells treated with selected compounds. (A) MDA-MB-231 cells treated with selected compounds for 24 hours. Medium replaced with MTT, then using a multi-label plate reader at 560 nm, cell viability measured. DMSO was acted as a negative control and DSF-Cu the positive control. The #39-Cu complex seemed potent with ~98% inhibition of cell proliferation (1.0-10.0 μM). (B) Structure of DSF-analogues made in a Chinese lab, #3, #18, #39. (C) MDA-MB-231 cells were treated with #3-Cu, #18-Cu, and #39-Cu in another MTT assay. DMSO acted as the negative control. #39-Cu identified as a potent anti-proliferative agent to MDA-MB-231 breast cancer cells.

Figure 2A shows the most potent compound-copper complex as #39-Cu, with ~100% inhibition of MDA-MB-231 cells at concentrations of 1 μM -10 μM, even stronger than DSF-Cu. Compound #3 was only a little less potent with ~98% inhibition at the highest concentration (10 μM), and ~90% inhibition at 5 μM. The rest of the compound-Cu complexes either had little to no affect on the cell proliferation of the MDA-MB-231 cells, when compared to the negative control, DMSO.

2. Anti-proliferative activity of selected structurally related DSF analogues

To investigate further upon the relationship of the structure of a compound and its ability to inhibit the proliferation of cells, three chemically related DSF-analogues #39, #3, and #18 were chosen for the next experiment with results exhibited in Figure 2b. The analogues #39, #3, and #18 were mixed with copper (II)-chloride, and color changes from light green to a dark green/ brown was observed in only the #39-Cu and #3-Cu mixtures, indicating the binding of the compound to copper, while #18 did not couple to Cu.
After the DSF-analogues #3, #18, and #39 were coupled to copper, another MTT assay was performed, using graded concentrations of each complex. Among the three mixtures tested, designated #39-Cu complex had most potency with ~98% inhibition of cell proliferation at concentrations 1.0 - 10.0 μM (Figure 2c). The #3-Cu complex had ~80% inhibition of cell proliferation at the high concentrations of 5.0 and 10.0 μM. The #18-Cu complex had no inhibition of cell proliferation, probably due to its incapability to bind to copper.

**Figure 3**

(A) The Chymotrypsin Activity of MDA-MB-231 Cells Treated with #3-Cu, #18-Cu, #39-Cu at Different Concentrations

(B) Ub proteins

(C) Protein images of DMSO, 0.5 μM, 1.0 μM, and 2.0 μM for #3-Cu, #18-Cu, and #39-Cu.

Fig.3. Proteasome inhibition and Apoptosis of #3-Cu, #18-Cu, and #39-Cu. (A) MDA-MB-231 cells were treated for 16 hrs with the compound - CuCl₂ mixtures of the compounds #39, #3, and #18 and incubated for 2 hours at 37°C in 100 μl assay buffer (10 mmol/l Tris–HCl, pH 7.5) with 10 μmol/l fluorogenic substrate Suc-LLVY-AMC (for proteasomal chymotrypsin-like activity) in the Isotemp Incubator. Activity was then measured with a multi-label counter at 380 nm. #39-Cu ( > 0.5 μM) proved to inhibit CT-like activity. (B) MDA/MB 231 Cells were treated with compound #39, #3, and #18 and CuCl₂ mixtures at concentrations of 0.5 μM, 1.0 μM, and 2.0 μM. Lysates were prepared, and SDS-PAGE gel was conducted. Specific antibodies for β-actin, PARP, BAX, and ubiquitin were used. Afterwards, protein amounts were visible on the autoradiography film with the enhanced chemiluminescence Reagent. #39-Cu had the most amount of ub. Proteins, BAX, and PARP cleavage, showing proteasome inhibition and cell death. (C) A Zeiss Axiovert 25 microscope was used for cell morphology of Petri dishes treated with different concentrations of the compound – CuCl₂ mixtures of compounds #39, #3, and #18 for 16 hrs. #39-Cu at 2.0 μM killed all the cells in the dish, indicating apoptosis. In all assays, DMSO was used as the negative control.
3. Morphological changes and molecular mechanisms associated with DSF analogues’ anti-proliferative effects

Using a dose-dependent strategy, MDA-MB-231 breast cancer cells were treated with the DSF-analogue-copper mixtures at concentrations of 0.5 $\mu$m, 1 $\mu$m, and 2 $\mu$m for 16 hrs. A CT-like activity assay and a Morphological assay were performed to measure proteasome inhibition and induction of apoptosis. Inhibition of the proteasomal CT-like activity was shown in the DSF-analogue #39-Cu complex as well as the #18-Cu complex in a dose dependent manner, indicating the most potent areas of apoptosis in those cells. The #39-Cu complex seemed to be the most potent in inhibiting the CT-like activity in concentrations as low as 1.0 and 2.0 $\mu$M (Figure 3a), consistent with its strong anti-proliferative effect in the MTT assay.

In the same experiment, morphological changes were observed in MDA-MB-231 cells when introduced to DSF analogues #39-Cu, #18-Cu, and #3-Cu. The morphological changes were seen in the typical shape of a cancer cell, which is usually long and thin, and which changed in dead cancer cells, which are circular. The #39-Cu complex killed 100% of the cancer cells in a concentration as low as 2.0 $\mu$M, and #18-Cu and #3-Cu killed some of the cancer cells at 0.5 $\mu$M to 2.0 $\mu$M concentrations (Figure 3c).

In the Western blot analysis, a dose-dependent accumulation of ubiquitinated proteins were observed for #39-Cu in all concentrations and #3-Cu at the concentration of 2.0 $\mu$M. In all concentrations, #39-Cu acquired a significant amount of ubiquitinated proteins, apart from the other complexes. It is also known that the Bax protein (p21/Bax) is cleaved by a protein, which produces a p18/Bax fragment and forms a homodimer p36/Bax (20, 21). We observed the presence of the p36/BAX in all compound-copper complexes, but a strong accumulation of p36/BAX in the cells treated with #39-Cu (2.0 $\mu$m), indicative of proteasome inhibition. MDA-MB-231 cells treated with #39-Cu in all concentrations had a dose dependent production of PARP cleavage fragment p65 and p89, which indicates caspase-3 and calpain activity, or the induction of apoptosis (Figure 3b). The high accumulation of Bax, PARP cleavage, and ubiquitinated proteins in cells treated with #39-Cu at a concentration of 2.0 $\mu$M indicate strong proteasomal inhibition. Proteasomal inhibition has occurred in the cells treated with #39-Cu at all concentrations, because Bax, Caspase 3 shown through PARP cleavage, and ubiquitinated proteins are all regularly target proteins for the proteasome. Beta-actin was used to ensure equal amount of protein loading.
4. The toxicity of #39-Cu on MCF 10A normal human breast cells

Figure 4

![Cell Viability of 10A Normal and MDA-MB-231 Cancer Cells when Treated with DSF-Cu and #39-Cu at 1.0 \( \mu M \)](image)

Fig. 4. The potency of the anti-proliferative effects and non-toxicity of #39-Cu when compared to DSF-Cu. MDA-MB-231 cells and 10A normal cells were treated with DSF-Cu, and #39-Cu. A MTT assay was performed, with DMSO as both negative and positive control, and cell viability measured. DSF-Cu and #39-Cu seemed to have similar toxicity levels to normal cells. #39-Cu had a more anti-proliferative effect on the cancer cells than DSF-Cu.

When 10A normal cells and MDA-MB-231 cells were treated with both DSF-Cu and #39-Cu at 1.0 \( \mu M \) concentration, #39-Cu had a more potent effect on killing MDA-MB-231 cells compared to DSF-Cu. At the same concentration of 1.0 \( \mu M \), the toxicity of both complexes to normal 10A cells is similar, with ~18-25% inhibition of normal cell growth (Figure 4).

Discussion:

In anti-cancer therapy, the main goal is to find a drug that successfully targets malignant cancer cells, while having minimal toxicity to normal cells. The DSF-copper complex has been shown in previous studies to be a potent complex that inhibits proteasomal activity and thereby induces apoptosis in human breast cancer cells in vitro and inhibits human breast cancer xenograft growth in vivo without obvious toxicity. Inhibiting proteasomal activity leads to apoptotic cell death, because the accumulation of ubiquitinated proteins and proteasome-target proteins leads to the induction of apoptosis.

DSF’s ability to bind to copper provides a strategy to only target cancer cells, since copper is essential for angiogenesis in tumor cells, which means copper helps metastatic cancer
cells grow and spread\textsuperscript{5-8, 17}. In contrast, normal cells have limited amounts of copper, which is under strict regulation, creating a differentiation between cancer and normal cells. In other studies, organic ligands and DSF were able to bind to copper, and form a potent complex that produced apoptosis in cancer cells\textsuperscript{3}. By being able to bind to copper in a cell and form an activated complex that helps kill that cell, these complexes are used to target cancer cells, which have an excess amount of copper that the complexes can bind to. However, it is well known that DSF itself can have severe toxicity and side effects\textsuperscript{19}. Therefore, DSF-analogues were tested in this current study in an attempt to find a more potent anti-proliferative agent in cancer cells while having less toxicity to normal cells.

In this study, a total of 34 DSF-analogues were screened. Then, designated analogues #3, #18, and #39 coupled with copper were chosen for further tests due to their potencies and their similar chemical structures. When the three DSF-analogues were mixed with copper, only #3 and #39 formed a compound-copper complex, indicated by the green to dark color change in the mixtures. Then, a MTT assay was performed to see if these analogues could produce cell death in MDA-MB-231, a metastatic cancer cell line. #3-Cu complex had strong inhibition of cell proliferation at 5.0 and 10.0 μM concentrations, #18-Cu complex had no inhibition of cancer cell growth, and #39 had ~98-100% inhibition of MDA-MB-231 cell proliferation. A chymotrypsin (CT) – like activity assay was performed, and in correspondence to the MTT assay results, cancer cells treated with #39-Cu had suppressed levels of CT-like activity from concentrations of 1.0 μM to 2.0 μM. These values predict even lower CT-like activity in higher concentrations with the #39-Cu complex. Inhibition of CT-like activity indicates the inhibition of the proteasome as well as induction of apoptosis, which is supported by the MTT assay of #39-Cu. To support the potency of #39-Cu in initiating cell apoptosis, a Western Blot assay was conducted, and #39-Cu treated cells were found to have the most accumulation of ubiquitinated proteins. All concentrations of #39-Cu had PARP cleavage, which shows cell death. At 2.0 μM, #39-Cu had a significant amount of BAX protein, which is known for inducing apoptosis, and a target protein of the proteasome. High accumulations of ubiquitinated proteins, BAX, and PARP in #39-Cu treated cells show proteasome inhibition and apoptosis, which is supported by the MTT and CT-like activity assay. Morphological changes were also seen most significantly in MDA-MB-231 cells, where the cancer cells were mostly dead. These multiple assays indicate the similarity between DSF-analogue #39-Cu complex and DSF-Cu complex in inducing apoptosis through proteasome inhibition, validating the first part of my hypothesis.

For the second part of my hypothesis, which was to test if any of the DSF-analogues had less toxicity to normal cells than DSF, DSF-Cu and #39-Cu were compared on its effects on both MDA-MB-231 breast cancer cells and 10A normal cells (Figure 4). The comparison showed #39-Cu having more inhibition of cell proliferation at a concentration of 1.0 μM when compared to DSF-Cu. Furthermore, at the same concentration, #39-Cu showed similar toxicity to 10A cells, when compared to the DSF-Cu complex.

As mentioned earlier, DSF primarily produces hepatotoxicity and neurotoxicity in patients. Therefore, this current study using 10A normal human breast cell lines for the toxicity evaluation may not be a good model. Instead, future studies are needed to test the toxicity of #39-Cu in normal liver and brain cell lines to determine if #39-Cu is a better anti-cancer drug than the DSF-Cu complex.
Conclusion:

The data presented here partially validates my hypothesis, and demonstrates that the designated DSF analogue #39 coupled with copper has more potent anti-proliferative effects in breast cancer cell lines when compared to DSF-Cu itself. However, the two complexes seem to have similar toxicity to normal human breast cells (MCF 10A), which are probably not the most optimal cellular model, given DSF’s other toxicities to the liver and brain. In conclusion, although more research on this topic is needed, the preliminary results suggest that the designated DSF analogue #39 is a promising compound as a novel anti-tumor agent.

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\[ \frac{1}{V} \int z \, dV = \frac{\pi r_1^2}{V H^2} \int_0^h \left( z^3 - 2z^2H + zH^2 \right) \, dz \]

\[ = \frac{\pi r_1^2}{V H^2} \left[ \frac{z^4}{4} - \frac{2z^3H}{3} + \frac{z^2H^2}{2} \right]_0^h \]

\[ = \frac{\pi r_1^2 h^4}{4 H^2} - \frac{2H}{3h} + \frac{H^2}{2h^2} \]

The circular cone is \( \frac{1}{3} \pi R^2 Z \), where the ratio of the height to the base radius is fixed. The cone's height and base radius are related by this ratio.
LINEARLY MANY FAULTS IN \((n, k)\)-STAR GRAPHS

ALLEN YUAN

1. Introduction

Parallel computing allows for high speed operation, but poses the problem of finding a fault resistant architecture for static interconnection networks; these networks are represented by graphs whose vertices corresponded to individual computers and whose edges corresponded to links between the processors. The earliest networks were structured as hypercubes. Later, the star graph was proposed by [1] and had the advantage of lower cost. However, the star graph has the disadvantage of having a factorial number of vertices, which creates large gaps in the number of possible vertices; [8] notes that the smallest star graph with at least 6000 vertices has 40,320 vertices. Several alternatives to the star graph have been proposed, such as the alternating group graphs [15], split stars [11], and arrangement graphs [13]. The alternative that we shall consider in this paper is the \((n, k)\)-star graph, a generalization of the star graph due to [12].

Many properties of interconnection networks are under study, such as topological properties, broadcasting issues, fault-tolerant Hamiltonian properties, and fault-tolerant routing. In particular, fault tolerance is of prime significance in interconnection networks, because the failure of computers in poorly structured networks can cause networks to disconnect and fail. It is of course desirable that the graph structures can remain connected after vertex deletion - clearly, however, even the deletion of vertices equal to the smallest degree of a vertex can, if properly chosen, always disconnect the graph. Other ways of evaluating the strength of networks were developed and examined in [2–4, 9, 11, 14, 18]. Since the graph cannot remain absolutely connected, it is desirable that when vertices are deleted, the remaining graph has a large component and small components with minimal numbers of vertices. That way, most of the network will still be connected, allowing the network to continue to function. The first
natural question to ask, then, is what will happen when the number of faults is approximately twice the smallest degree, as was examined in [6, 7, 9, 10, 19]. Then, it was explored what happens when any linear number of vertices are deleted for hypercubes in [20] and Cayley graphs generated by transpositions in [6].

This paper examines the fault resistance of \((n, k)\)-stars when small numbers of vertices are deleted and generalizes to the case of any linear number of vertices being deleted. The main result is for a bound in terms of positive integers \(n, k, \) and \(r\) on the greatest number of vertices that can be deleted from an \((n, k)\)-star such that the graph must still be either connected or have a large component and some number of small components with at most \(r - 1\) vertices in total.

2. Definitions and Preliminaries

A graph \(H = (V, E)\) with vertex set \(V\) and edge set \(E\) is \(r\)-regular if the degree of every vertex of \(H\) is \(r\). If \(W \subset V\) is a set of vertices of \(H\), the graph obtained by deleting the vertices of \(W\) from \(H\) will be denoted by \(H - W\). A noncomplete graph \(H\) is \(r\)-connected if deleting any set of less than \(r\) vertices results in a connected graph. A complete graph with \(r + 1\) vertices is \(k\)-connected for \(k \leq r\). An \(r\)-regular graph is maximally connected if it is \(r\)-connected.

The \((n, k)\)-star graph, denoted \(S_{n,k}\), is defined for positive integers \(n\) and \(k\) such that \(n > k \geq 2\). The vertex set of the graph is all the permutations on \(k\) elements of the set \(\{1, 2, \ldots, n\}\). Two vertices corresponding to the permutations \([a_1, a_2, \ldots, a_k]\) and \([b_1, b_2, \ldots, b_k]\) are adjacent if and only if either:

1. There exists an integer \(2 \leq s \leq k\) such that \(a_1 = b_s\) and \(b_1 = a_s\) and for any \(i \neq s, 1 < i \leq k\), we have \(a_i = b_i\).
2. For all \(2 \leq i \leq k\), we have \(a_i = b_i\) and \(a_1 \neq b_1\).

See Figures 1 and 2 for \(S_{4,2}\) and \(S_{4,3}\), respectively. Note that \(S_{n,n-1}\) is just the \(n\)-dimensional star graph (as defined in [1]). We present a few preliminary results from previous research.
The following two structural facts, due to [6] and [7], respectively, were proven for a more general graph, but can be simplified as stated for $S_{n,n-1}$.

**Theorem 2.1.** Let $G$ be the $n$-dimensional star graph. If $T$ is a set of vertices of $G$ with $|T| \leq k(n - 1) - \frac{k(k+1)}{2}$, where $1 \leq k \leq n - 2$, then $G - T$ is either connected or has a large connected component and small components having at most $k - 1$ vertices in total.

**Theorem 2.2.** Let $G$ be the $n$-dimensional star graph and $T$ be a set of vertices of $G$ such that $|T| \leq 3n - 8$. $G - T$ is either connected or has a large component and small components with at most two vertices in total.

Let the set of vertices representing permutations whose $k$th element is $i$ be $H_i$ for $1 \leq i \leq n$. In this paper, a set of vertices to be deleted will be denoted as $T$. Define $T \cap H_i = T_i$ and $|T_i| = t_i$ for $1 \leq i \leq n$. This notation will be used throughout the paper. It can easily be seen that $S_{n,k}$ is $(n-1)$-regular as each vertex has $k-1$ neighbors by adjacency rule (1) and $n-k$ neighbors by adjacency rule (2). Let us first note some other preliminary facts about $S_{n,k}$.

1. $H_i$ is isomorphic to $S_{n-1,k-1}$ when $n > k > 2$. This is clear from noting that removing $i$ from all the permutations in $H_i$ results in permutations of $k-1$ elements from $\{1, 2, \cdots, n\} - \{i\}$. This fact is highly useful in the inductive proofs of the paper, as we can often use the induction hypothesis on the $H_i$. 
(2) $S_{n,k}$ has $\frac{n!}{(n-k)!}$ vertices, which is seen from a simple count of the number of permutations of $k$ elements from an $n$-element set. It follows that $H_i$ has $\frac{(n-1)!}{(n-k)!}$ vertices for all $1 \leq i \leq n$.

(3) Each vertex in $H_i$ has a unique neighbor outside of $H_i$. Consider some vertex representing the permutation $[a_1, a_2, \cdots, a_{k-1}, i]$. Any neighbor outside $H_i$ must have a different last element, and thus must apply adjacency rule (1) with $s = k$, giving the unique neighbor as $[i, a_2, a_3, \cdots, a_{k-1}, a_1]$. It follows that there are exactly $\frac{(n-1)!}{(n-k)!}$ edges between $H_i$ and $S_{n,k} - H_i$.

(4) For each pair $H_i$ and $H_j$, there are exactly $\frac{(n-2)!}{(n-k)!}$ independent edges (that is, edges such that no two are incident on a common vertex) between them. Note that all edges from $H_i$ to $H_j$ have to result from adjacency rule (1) where permutations of the form $[j, a_2, a_3, \cdots, a_{k-1}, i]$ are connected to permutations of the form $[i, a_2, a_3, \cdots, a_{k-1}, j]$. Thus, the number of such pairs is the number of ways to choose $a_2$ through $a_{k-1}$, which is the number of $k - 2$ element permutations on the set $\{1, 2, \cdots, n\} - \{i, j\}$, or $\frac{(n-2)!}{(n-k)!}$. Clearly, the edges are independent.

3. Precise results for small vertex deletions

[8] proves the following theorem about $(n, k)$-stars.

**Theorem 3.1.** $S_{n,k}$ is maximally connected for any $n > k \geq 2$.

Given this result about the connectivity of the graph when $n - 2$, approximately $n$, vertices are deleted, it is natural to then consider when approximately $n + k$ vertices are deleted, leading to the following theorem. We are considering when $k$ more vertices are added instead of $n$ vertices because it can easily be seen that large numbers of vertices can be disconnected from $S_{n,2}$ with relatively small vertex deletions in comparison to those needed to disconnect comparable numbers of vertices in $S_{n,n-1}$, implying a dependence on $k$.

**Theorem 3.2.** Let $n$ and $k$ be positive integers such that $n > k \geq 2$. Let $T$ be a subset of the vertices of $S_{n,k}$ such that $|T| \leq n + k - 4$. Then, $S_{n,k} - T$ is either connected or has a large component and a singleton (single vertex).
Proof. Define $H_i$’s, $T_i$’s, $t_i$’s as usual. We proceed by induction on $k$. First, when $k = 2$, the graph is connected by Theorem 3.1. We consider the base case $k = 3$ of removing $n - 1$ vertices from $S_{n,3}$.

**Case 1:** $t_i \leq n - 3$ for all admissible $i$. Then, every $H_i - T_i$ is still connected because $S_{n-1,2}$ is maximally connected. There exists $l$ such that $t_l = 0$ because only $n - 1$ vertices are to be deleted and there are $n$ choices for $l$. Then, for any $i \neq l$, a total of $t_i + t_l \leq n - 3$ vertices are deleted from $H_i \cup H_l$, and since $H_i$ and $H_l$ have $\frac{(n-2)!}{(n-3)!} = n - 2$ independent edges between them, one edge must remain. Thus, since $i$ was arbitrarily chosen, there is an edge between $H_i - T_i$ and $H_l - T_l$ for all $1 \leq i \leq n$ so $S_{n,3}$ is connected.

**Case 2:** $t_i > n - 3$ for some $i$. Then, at most one vertex is removed from $S_{n,k} - H_i$, so $S_{n,k} - T$ has a component $Y$ that contains every $H_j - T_j$ except possibly components of $H_i - T_i$. However, suppose at least two vertices of $H_i$ are not in $Y$. Then, before the vertex deletion, each had a neighbor outside of $H_i$, so each of these outside neighbors must be in $T$, implying that at least two vertices outside of $H_i$ are deleted, a contradiction. Thus, at most one vertex is not in $Y$ and the base case is proved.

Now suppose that the statement holds for all pairs $(n, k)$ such that $k < k'$ and $2 \leq k < n$ for some $k'$ at least 4, and consider deleting at most $n + k' - 4$ vertices from $S_{n,k'}$ where $n > k'$. Again, we have two cases:

**Case 1:** $t_i \leq n - 3$ for all admissible $i$. Then, every $H_i - T_i$ is connected. As before, since there are $\frac{(n-2)!}{(n-k')!} \geq (n-2)(n-3) > 2(n-2) > n + k' - 4$ independent edges (recall that $n > k' \geq 4$) between $H_i$ and $H_j$ for any $1 \leq i < j \leq n$, but only $n + k' - 4$ total deleted vertices, one edge must remain between every pair $H_i - T_i$ and $H_j - T_j$, so $S_{n,k} - T$ is connected.

**Case 2:** $t_i > n - 3$ for some $i$. Suppose there is $j \neq i$ for which $t_j > n - 3$. Then, $n + k' - 4 = |T| \geq t_j + t_i \geq 2n - 4 > n + k' - 4$, a contradiction. Thus, $t_j \leq n - 3$ for every $j \neq i$, implying $H_j - T_j$ is connected for every $j \neq i$; in fact, they are part of one large component, say $Y$, in $S_{n,k} - T$ by similar reasoning as in Case 1. If $t_i \leq (n - 1) + (k' - 1) - 4 = n + k' - 6$, then by the inductive hypothesis, $H_i - T_i$ is either connected, in which case we are done as
in Case 1, or has two components, one of which is a singleton. Since at most \( n + k' - 6 \) vertices were removed from \( H_i \) and there is only one vertex in the small component, the large component has at least

\[
\frac{(n - 1)!}{(n - k')!} - (n + k' - 6) - 1 \geq (n - 1)(n - 2)(n - 3) - n - k' + 5
\]

\[
\geq (n - 1) \cdot 3 \cdot 2 - n - k' + 5 > 2n
\]

vertices, and in turn, at least \( 2n \) independent edges going out. Since \( |T| - |T_i| < n + k' - 4 < 2n \), the large component of \( H_i - T_i \) is part of \( Y \) so at most one vertex is not part of \( Y \) as desired. If \( t_i = n + k' - 5 \) or \( t_i = n + k' - 4 \), the same argument as in Case 2 of the base case applies, as only one or zero vertices outside of \( H_i \) can be deleted. Hence, our induction is complete. □

This bound is best possible. Consider the two vertices corresponding to the permutations \([1, n - k + 2, n - k + 3, \ldots, n]\) and \([2, n - k + 2, n - k + 3, \ldots, n]\), which exist since \( n - k + 2 \geq 3 \).

The vertices obtained by applying adjacency rule (1) with the transposition \((1, j)\) for \( 2 \leq j \leq k \) to each of these two permutations give \( k - 1 \) distinct permutations per vertex, for a total of \( 2k - 2 \) permutations. The vertices obtained by adjacency rule (2) give \( n - k - 1 \) choices for the first element in the permutation, and so \( n - k - 1 \) permutations. Thus, there are a total of \((2k - 2) + (n - k - 1) = n + k - 3\) distinct vertices in the neighbor sets of the two chosen permutations. Hence, if we delete \( n + k - 3 \) vertices, we can disconnect two vertices, proving that the bound of \( n + k - 4 \) in Theorem 3.2 is best possible.

Now that we have produced a bound for the maximum size of a small component in the case of approximately \( n + k \) vertices, the natural question is to examine when \( k \) more, or approximately \( n + 2k \) vertices are deleted.

**Theorem 3.3.** Let \( n \) and \( k \) be positive integers such that \( n > k \geq 2 \). Let \( T \) be a subset of the vertices of \( S_{n,k} \) such that \(|T| \leq n + 2k - 6\). Then, \( S_{n,k} - T \) is either connected or has a large component and small components with at most two vertices in total.
Proof. When \( k = n - 1 \), we have a star graph and so after removal of at most \( n + 2k - 6 = 3n - 8 \) vertices, by Theorem 2.2, the desired conclusion holds; thus, consider when \( n - 1 > k \). Now, define \( T_i's, t_i's, H_i's \) as before and proceed by induction on \( k \), and again the \( k = 2 \) case follows by Theorem 3.1. First, we consider the base case \( k = 3 \) of removing \( n \) vertices from \( S_{n,3} \).

There are two cases:

**Case 1:** \( t_i \leq n - 3 \) for all admissible \( i \). Then, all of the \( H_i - T_i \) are still connected because \( S_{n-1,2} \) is maximally connected. Note that as in the proof of Theorem 3.2, for any \( H_i \) and \( H_j \) \((i \neq j)\), there are \( n - 2 \) edges between them, so an edge remains between \( H_i - T_i \) and \( H_j - T_j \) unless \( t_i + t_j \geq n - 2 \). Thus, if \( t_i = 1 \) for all admissible \( i \), then all the \( H_i - T_i \) are part of one large component. Otherwise, since \( \sum_{i=1}^{n} t_i \leq n \), there exists some \( l \) such that \( t_l = 0 \). Then, for any \( i \neq l, t_i + t_l < n - 2 \), so there is an edge between \( H_i - T_i \) and \( H_l - T_l \); since \( i \) was arbitrarily chosen, there is an edge between \( H_i - T_i \) and \( H_l - T_l \) for every \( i \neq l \) and thus, \( S_{n,3} - T \) is still connected.

**Case 2:** \( t_i > n - 3 \) for some \( i \). Since at most two vertices are removed from \( S_{n,k} - H_i \), so there is a component \( Y \) in \( S_{n,k} - T \) containing every \( H_j - T_j \) except possibly components of \( H_i - T_i \). Suppose at least three vertices of \( H_i \) are disconnected from the rest of the graph. Then, before the vertex deletion, each had a neighbor outside of \( H_i \), so each of these neighbors must be in \( T \), implying that \( |T| - |T_i| \geq 3 \), a contradiction. Thus, at most two vertices are disconnected and the base case is proven.

Now suppose that the statement holds for all pairs \((n, k)\) such that \( k < k' \) and \( 2 \leq k < n \) for some \( k' \) at least 4, and consider deleting at most \( n + 2k' - 6 \) vertices from \( S_{n,k'} \) where \( n - 1 > k' \).

**Case 1:** \( t_i \leq n - 3 \) for all admissible \( i \). Then, every \( H_i - T_i \) is connected. Furthermore, between any pair of \( H_i - T_i \) and \( H_j - T_j \), there are \( \frac{(n-2)!}{(n-k)!} \geq (n-2)(n-3) \) independent edges. However, this is greater than the number of vertices deleted because \( n - 1 > k' \geq 4 \) implies \( n \) is at least 6 from which it follows that \( (n-2)(n-3) \geq 3(n-2) > n + 2k' - 6 \). Thus, there is an edge between \( H_i - T_i \) and \( H_j - T_j \) for any pair \( i, j \), so \( S_{n,k} - T \) is connected.
Case 2: $t_i > n - 3$ for exactly one $1 \leq i \leq n$. This implies that for $j \neq i$, $H_j - T_j$ is connected, and moreover, every such $H_j - T_j$ belongs to one large component $Y$ in $S_{n,k} - T$. If $t_i > n + 2k' - 9$, then $|T - T_i| \leq 2$, and so as in Case 2 of the base case, at most two vertices are not in $Y$. If $t_i \leq n + 2k' - 9 = (n-1) + 2(k' - 1) - 6$, then we may apply the inductive hypothesis, from which it follows that $H_i - T_i$ is either connected or has a large component and small components having at most two vertices in total. If it is connected, we are done in the same way as Case 1, where $S_{n,k} - T$ is connected. Otherwise, note that the large component must be part of $Y$ because the number of edges out of $H_i$, $\frac{(n-1)!}{(n-k')!} \geq (n-1)(n-2)(n-3)$, is greater than the number of vertices deleted plus the number of vertices in the small component, which is at most $n + 2k' - 6 + 2 < 3n < (n-1) \cdot 3 \cdot 2 < (n-1)(n-2)(n-3)$. Hence, at most two vertices are disconnected from $Y$, as desired.

Case 3: $t_i > n - 3$ for more than one value $1 \leq i \leq n$. Suppose there exist $i, j, l$ distinct positive integers such that $t_i, t_j, t_l > n - 3$. Then,

$$n + 2k' - 6 \geq |T| \geq t_i + t_j + t_l \geq 3(n - 2) > n + 2k' - 6$$

a contradiction. Thus, we need only consider when there are exactly two positive integers, $i$ and $j$, such that $t_i, t_j > n - 3$, and so $H_i - T_i$ is connected for $l \neq i, j$. Furthermore, there is a component $Y$ in $S_{n,k} - T$ containing all the $H_l - T_l$ for $l \neq i, j$ because each pair of such $H_l - T_l$ has fewer than $n - 2$ vertices deleted in the pair. If one of $t_i$ and $t_j$ is greater than $(n-1) + (k' - 1) - 4$, then

$$t_i + t_j \geq (n-1) + (k' - 1) - 3 + (n-2) = 2n + k' - 7 > n + 2k' - 6.$$ 

This is a contradiction since at most $n + 2k' - 6$ vertices are deleted. Thus, $t_i, t_j \leq (n-1) + (k' - 1) - 4$. Hence, by Theorem 3.2 on $H_i - T_i$ and $H_j - T_j$, each has at most one vertex disconnected from $Y$. Furthermore, by the same argument as in Case 2, the large components of $H_i - T_i$ and $H_j - T_j$ are part of $Y$, so at most two vertices are not in $Y$ and our induction is complete. □
This bound is also best possible. For graphs which are not star graphs, consider the three vertices corresponding to the permutations $[1, n-k+2, n-k+3, \ldots, n]$, $[2, n-k+2, n-k+3, \ldots, n]$, and $[3, n-k+2, n-k+3, \ldots, n]$, which exist since $n-k+2 \geq 4$. The vertices obtained by applying adjacency rule (1) with the transposition $(1, j)$ for $3 \leq j \leq k$ to each of these three permutations give $k-1$ distinct permutations per vertex, for a total of $3k-3$ permutations. The vertices obtained by adjacency rule (2) give $n-k-2$ choices for the first element in the permutation, and so $n-k-2$ permutations. Thus, there are a total of $(3k-3) + (n-k-2) = n+2k-5$ vertices in the union of the neighbor sets of the three chosen permutations. Hence, if we delete $n+2k-5$ vertices, we can disconnect three vertices, proving that the bound of $n+2k-6$ in Theorem 3.2 is best possible in non-star graphs. Now consider the star graph, when $n = k-1$, and we show that we can disconnect a path of length 2 by removing $3n-7$ vertices. Each vertex has degree $n-1 \geq 2$; this immediately implies that there exists a path of length 2, so consider any such path with vertices $x$, $y$, and $z$ (in that order). Each of $x$ and $z$ have $n-2$ neighbors off the path, and $y$ has $n-3$ neighbors not on the path. Thus, the union of the neighbor sets of $x$, $y$, and $z$ (not including the path itself) has size at most $2(n-2) + (n-3) = 3n-7$. Therefore, deleting these vertices disconnects the path from the graph, as desired. Hence, our bound of $n+2k-6$ is best possible.

4. General results for larger vertex deletions

The previous two results took a very similar form, where case analysis and induction were used to obtain tight results. One wonders whether this can be done in general, to find a result when approximately $n + (r-1)k$ vertices are deleted - the pattern seems to be that deleting at most $n + (r-1)k - 2r$ vertices will result in small components of at most size $r-1$ in total. It can be noticed, however, that the proof of Theorem 3.3 was already significantly more complex than that of Theorem 3.2; thus, to generalize to get precise results for larger deletions may be difficult; indeed, the statement does not even hold in all cases - for example, when $r$ is very large, we can delete all of the $\frac{(n-1)!}{(n-k)!}$ independent edges coming out of $H_i$ and disconnect $H_i$, which has $\frac{(n-1)!}{(n-k)!}$ vertices. However, in many cases, this tight bound is still
possible, as will be proven in Theorem 4.2. In general, though, to balance out the strength of the bound and the ease of proving such a bound, we will instead give a bound in the form

\[ n + (r - 1)k - f(r) \]

for some function \( f \), as is shown in the next result. This will make the bound asymptotically tight for large \( n \).

**Theorem 4.1.** Let \( n, k, \) and \( r \) be positive integers such that \( n > k \geq 2 \) and \( r \geq 1 \). If \( T \) is a set of vertices of \( S_{n,k} \) such that \( |T| \leq n + (r - 1)k - r(r - 1) - 2 \), then \( S_{n,k} - T \) is either connected or has a large component and small components with at most \( r - 1 \) vertices in total.

**Proof.** For ease of notation, let \( F(x) = x(x - 1) + 2 \). We proceed by induction on \( r \). The \( r = 1 \) case is true because \( S_{n,k} \) is maximally connected. For \( r = 2 \) and \( r = 3 \), the result follows trivially from Theorems 3.2 and 3.3, respectively. Now suppose \( r \geq 4 \). We have that for \( k = 2, 3, 4 \), since \( r \geq 4 \), \( n + (r - 1)k - r(r - 1) - 2 \leq n - 2 \) from which it follows that \( S_{n,k} \) is connected by Theorem 3.1, so suppose now \( k \geq 5 \) and assume that for some \( r' \), the theorem holds for all \( 1 \leq r < r' \). We aim to show that it also holds for \( r = r' \).

To prove this, we set up another induction on \( k \). We have already checked the base cases when \( k < 5 \), so suppose there is some \( k' \geq 5 \) such that the statement is true for all \( k < k' \). It suffices to show the statement for \( k = k' \). Define \( H_i \)'s, \( T_i \)'s, \( t_i \)'s as usual.

First, in the case that \( n = k' + 1 \), \( S_{n,k'} \) is a star graph and we delete at most \( n + (r' - 1)k' - F(r') = n + (r' - 1)(n - 1) - r'(r' - 1) - 2 < r'n - \frac{r'(r'+1)}{2} \) vertices. The result then follows from Theorem 2.1; assume, then that \( n \geq k' + 2 \). Now note that if \( r' \geq n - 1 \), then

\[
\begin{align*}
n + (r' - 1)k' - r'(r' - 1) - 2 &< n + (r' - 1)(k' - r') \\
&\leq n + (r' - 1)(n - 2 - r') \\
&\leq n + 1 - r' \leq n - 3.
\end{align*}
\]

Thus, at most \( n - 3 \) vertices will be deleted and \( S_{n,k'} - T \) is connected, as desired. Therefore, we only need to consider when \( n - 1 > r' \). We have two cases:
Case 1: \( t_i > (n - 1) + (r' - 1)(k' - 1) - F(r') \) for some \( 1 \leq i \leq n \). Then, \( S_{n,k'} - H_i \) has vertices deleted. Since the \( H_j \)'s are maximally connected, \( H_j - T_j \) is connected for every \( j \neq i \); we now show they are all part of the same component \( Y \) in \( S_{n,k'} - T \). Note that each pair of two \( H_j - T_j \) (\( j \neq i \)) have \( \frac{(n-2)!}{(n-k')!} > n-2 > r'-1 \) edges between them, so one edge must remain after deletion of at most \( r'-1 \) vertices; thus, the pair is connected, and thus every \( H_j - T_j \) is part of \( Y \) for \( j \neq i \). If \( H_i - T_i \) is connected, then we are done. Now suppose that at least \( r' \) vertices of \( H_i - T_i \) are not in \( Y \). However, every vertex in \( H_i \) is connected to a unique vertex outside of \( H_i \), and thus, since at most \( r' - 1 \) vertices outside of \( H_i \) are in \( T \), it is possible to choose a vertex not in \( Y \) whose unique neighbor outside of \( H_i \) is not in \( T \). This chosen vertex is then not disconnected from \( Y \), a contradiction.

Case 2: \( t_i \leq (n - 1) + (r' - 1)(k' - 1) - F(r') \) for all \( 1 \leq i \leq n \). We will first show that the big components of all the \( H_i - T_i \) are all part of one component \( Y \) in \( S_{n,k'} - T \). First, we show that at least one \( H_i - T_i \) is connected; suppose \( H_i - T_i \) is disconnected for all \( 1 \leq i \leq n \). Then, \( t_i \geq n - 2 \) for all admissible \( i \), and so the total number of deleted vertices is at least

\[
(n(n-2) > 1 + (n-2)^2 + (n-1) > 1 + (r' - 1)(n-2) + (n-1) > 1 + (r' - 1)(k' - 1) + (n-1) > (r' - 1)(k' - r') + n > n + (r' - 1)k' - F(r').
\]

This is a contradiction because the last number is the greatest number of vertices that could be deleted. Thus, there exists some \( l \) such that \( H_l - T_l \) is connected. Now consider any \( H_i - T_i \). Since \( t_i \leq (n - 1) + (r' - 1)(k' - 1) - F(r') \), we have, by the inductive hypothesis on \( n-1 \), \( r' \), and \( k' - 1 \), that there are at most \( r' - 1 \) vertices in the small components of \( H_i - T_i \). As long as the total number of vertices in the small components plus the number of deleted vertices is smaller than the total number of edges originally between \( H_i \) and \( H_l \), then the big components
of $H_i - T_i$ and $H_l - T_l$ will be connected. Hence, we require $r' - 1 + n + (r' - 1)k' - F(r') < \frac{(n-2)!}{(n-k')!}$.

However, $k' > 4$ so $\frac{(n-2)!}{(n-k')!} > (n-2)(n-3)$. But

$$r' - 1 + n + (r' - 1)k' - F(r') = r' - 1 + n + (r' - 1)k' - (r' - 1)r' - 2 < n + (r' - 1)(k' - 2)$$

$$\leq n + (r' - 1)(n - 4) = n + (n - 3)(n - 4) \leq (n - 3)(n - 2)$$

as desired. The last inequality is because $n \geq 6$, since $n > k' \geq 5$. Thus, since the choice of $i$ was arbitrary, the large component of $H_i - T_i$ for every admissible $i$ is connected to $H_l - T_l$, and thus, all these large components are part of $Y$. Now, we show that the total number of vertices in the small components is at most $r' - 1$. Suppose the contrary, that at least $r'$ vertices are in the small components.

Define the function $g(x) = (n - 1) + (x - 1)(k' - 1) - F(x)$. For any $i$ such that $H_i - T_i$ is disconnected, let $q_i$ denote the total number of vertices in the small components of $H_i - T_i$. As noted earlier, at most $r' - 1$ vertices are disconnected in any $H_i - T_i$, so $q_i \leq r' - 1$. Thus, at least two of the $q_i$ are positive since the sum of the $q_i$ is at least $r'$. Hence, without loss of generality (we will not use any facts that are affected by a particular ordering of the $q_i$), we may let $q_1, q_2, \ldots, q_j$ be at least 1 where $j \geq 2$. By the induction hypothesis, we have that if $t_i \leq g(x)$, then no more than $x - 1$ vertices are in the small components of $H_i$ for any integer $x \leq r'$. Thus, for all integers $m \leq q_i$, we have that $t_i > g(m)$. Otherwise, $t_i \leq g(m)$ and at most $m - 1 \leq q_i - 1$ vertices are in the small components of $H_i - T_i$, contradicting the fact that the small component is defined to have $q_i$ vertices. Thus, since $\sum_{i=1}^{n} q_i \geq r'$, we can choose numbers $p_i$ for $1 \leq i \leq j$ such that $\sum_{i=1}^{j} p_i = r'$ and $p_i \leq q_i$ for all admissible $i$. The latter of these two implies that for $1 \leq i \leq j$, we have $t_i > g(p_i)$.

The sum of the $t_i$ for $1 \leq i \leq j$ is at most the total number of deleted vertices, so

$$n + (r' - 1)k' - F(r') \geq \sum_{i=1}^{j} t_i \geq \sum_{i=1}^{j} (g(p_i) + 1) = \sum_{i=1}^{j} (n + (p_i - 1)(k' - 1) - F(p_i))$$

$$= jn + (k' - 1)\sum_{i=1}^{j} (p_i - 1) - \sum_{i=1}^{j} F(p_i).$$
Recall that \( \sum_{i=1}^{j} p_i = r' \) which implies \( \sum_{i=1}^{j} (p_i - 1) = r' - j \) so plugging this in, we have

\[
 n + (r' - 1)k' - F(r') \geq jn + (k' - 1)(r' - j) - \sum_{i=1}^{j} F(p_i).
\]

Equivalently,

\[
 \sum_{i=1}^{j} F(p_i) - F(r') - r' \geq (j - 1)(n - k') + j.
\]

Note that for any positive integers \( a \) and \( b \), \( F(a) + F(b) \leq F(a + b - 1) + F(1) \) because \( F(a + b - 1) + F(1) - F(a) - F(b) = 2(a - 1)(b - 1) \geq 0 \). Thus, for any two of the \( p_i \), we can define an operation as changing one to have value 1 and the other to have value one less than the sum of the original two, and the value \( \sum_{i=1}^{j} F(p_i) \) does not decrease under the operation. Hence, if we do this operation \( j - 1 \) times, pairing each \( p_2 \) through \( p_j \) with \( p_1 \), we can transform the \( p_i \) into a sequence \( s_i \) for \( 1 \leq i \leq j \) such that \( \sum_{i=1}^{j} F(s_i) \geq \sum_{i=1}^{j} F(p_i) \) and \( s_i = 1 \) for \( 2 \leq i \leq j \). Note that the sum of all the arguments is invariant under this operation, so \( s_1 = \sum_{i=1}^{j} p_i - \sum_{i=2}^{j} s_i = r' - (j - 1) \). Hence, we have \( \sum_{i=1}^{j} F(s_i) = F(r' - (j - 1)) + 2(j - 1) \) (since \( F(1) = 2 \) and \( s_i = 1 \) for \( 2 \leq i \leq j \)). Thus,

\[
 \sum_{i=1}^{j} F(p_i) - F(r') + r' \leq \sum_{i=1}^{j} F(s_i) = F(r' - (j - 1)) + 2(j - 1) - F(r') + r' = (r' - j + 1)(r' - j) + 2(j - 1) - r'(r' - 1) + r' = (r' - j + 1)(r' - j) + 2(j - 1) - r'(r' - 1) + r'.
\]

We aim to show this is actually less than \( (j - 1)(n - k') + j \), which would contradict the statement above that \( \sum_{i=1}^{j} F(p_i) - F(r') + r' \) is at least \( (j - 1)(n - k') + j \). Thus, we wish to show

\[
 (r' - j + 1)(r' - j) + 2(j - 1) - r'(r' - 1) + r' < (j - 1)(n - k') + j.
\]

We can write equivalently that

\[
 (r' - j + 2)(r' - j) < (j - 1)(n - k' - 2) + r'(r' - 1).
\]

However, this follows from the facts that \( j \geq 2 \) and \( n \geq k' + 2 \) (since we already solved the case of a star graph), which imply that \( 0 \leq (j - 1)(n - k' - 2) \) and \( (r' - j + 2)(r' - j) < r'(r' - 1) \), producing the desired contradiction and completing our induction.
As mentioned before, the above bound can be significantly tightened in many cases to a bound that is best possible.

**Theorem 4.2.** Let \( n, k, \) and \( r \) be positive integers such that \( n > k \geq 2 \) and \( n-k+1 \geq r \geq 1 \). If \( T \) is a set of vertices of \( S_{n,k} \) such that \(|T| \leq n + (r-1)k - 2r\), then \( S_{n,k} - T \) is either connected or has a large component and small components with at most \( r - 1 \) vertices in total.

**Proof.** Much of this proof is similar to that of Theorem 4.1. Define \( H_i \)'s, \( T_i \)'s, \( t_i \)'s as usual. First, the \( k = 2 \) case follows from Theorem 3.1. We establish the case \( k = 3 \) when we have \( n-2 \geq r \) and we delete \( n + r - 3 \) vertices. There are two cases:

**Case 1:** \( t_i \leq n-3 \) for all \( 1 \leq i \leq n \). Then, every \( H_i - T_i \) is connected. Note that for every pair of distinct integers \((l, j)\), there are \( n-2 \) edges between \( H_i \) and \( H_j \). Thus, if \( t_l + t_j < n-2 \), then there is an edge between \( H_l - T_l \) and \( H_j - T_j \). If \( t_i = n-3 \) for some \( i \), then note that we cannot have \( t_j \) positive for all \( j \neq i \), since then, \( \sum_{j=1}^{n} t_j \geq (n-1) + t_i = 2n - 4 > n + r - 3 \).

Thus, there exists \( l \neq i \) such that \( t_l = 0 \). Then, for any other \( j \neq l \), we have that \( t_j + t_l < n-2 \) so there is an edge between \( H_j - T_j \) and \( H_l - T_l \) for all \( j \neq l \) and so \( S_{n,3} - T \) is connected. If \( t_i < n-3 \) for all \( 1 \leq i \leq n \), then there exists \( l \) such that \( t_l < 2 \), or else \(|T| \geq 2n > n + r - 3\), a contradiction. Consider this \( t_l \). For every \( j \neq l \), \( t_l + t_j < n-2 \), and so there is an edge between \( H_j - T_j \) and \( H_l - T_l \) for every \( j \neq l \), implying \( S_{n,3} - T \) is connected.

**Case 2:** \( t_i > n-3 \) for some \( 1 \leq i \leq n \). Then, at most \( r-1 \leq n-3 \) vertices are deleted outside of \( H_i \), and thus, there is a component \( Y \) in \( S_{n,3} - T \) containing every \( H_j - T_j \) for \( j \neq i \). Suppose that at least \( r \) vertices of \( H_i - T_i \) are not in \( Y \). Then, since each vertex in \( H_i \) has a unique neighbor outside \( H_i \), it follows from the fact that at most \( r-1 \) vertices are deleted outside \( H_i \) that there exists a vertex whose neighbor outside of \( H_i \) was not deleted, and thus, this vertex is not disconnected from \( Y \). Thus, at most \( r-1 \) vertices are not in \( Y \), completing the \( k = 3 \) case.

Now, we can assume \( k \geq 4 \). As before, we induct on \( r \). The \( r = 1, 2, 3 \) cases follow from Theorems 3.1, 3.2, and 3.3, respectively, so assume we have the statement is true for all \( r < r' \)
for some $4 \leq r' \leq n - k + 1$. We will proceed by induction on $k$ to prove the case $r = r'$. The base cases $k = 2, 3$ are established above, so assume the statement holds for all $k < k'$ for some $k' \geq 4$ and consider when $n + (r' - 1)k' - 2r'$ vertices are deleted from $S_{n,k'}$. We have two cases:

**Case 1:** $t_i > (n - 1) + (r' - 1)(k' - 1) - 2r'$ for some $1 \leq i \leq n$. Then, $|T - T_i| \leq r' - 1 \leq n - k' \leq n - 3$. Thus, there exists a component $Y$ in $S_{n,k'} - T$ containing all vertices except possibly those in $H_i - T_i$. As before, if at least $r'$ vertices in $H_i - T_i$ are not in $Y$, then since each vertex in $H_i$ has a unique neighbor outside of $H_i$, it follows that one of these $r'$ vertices is connected to $Y$, a contradiction. Thus, at most $r' - 1$ vertices are not in $Y$, as desired.

**Case 2:** $t_i \leq (n - 1) + (r' - 1)(k' - 1) - 2r'$ for all $1 \leq i \leq n$. We will prove much of this case by showing that each of the key bounds in the second case of the inductive step in the proof of Theorem 4.1 still hold when $F(x)$ is redefined as $2x$ and $n - k' + 1 \geq r'$.

We first needed the bound $n(n - 2) > n + (r' - 1)k' - F(r')$. This still holds, as

$$n + (r' - 1)k' - F(r') < n + r'(k' - 2) \leq n + (n - k' + 1)(k' - 2)$$

$$\leq n + n(n - 3) = n(n - 2).$$

Thus, as before, there exists some $l$ such that $H_i - T_i$ is connected. We can still use the inductive hypothesis on $n - 1$, $r'$, and $k' - 1$, because $(n - 1) - (k' - 1) + 1 = n - k' + 1 \geq r'$; thus, there are at most $r' - 1$ vertices in the small components of $H_i - T_i$ for any $1 \leq i \leq n$. Then, we required $r' - 1 + n + (r' - 1)k' - F(r') < \frac{(n - 2)!}{(n - k')!}$. However, $k' \geq 4$ so $\frac{(n - 2)!}{(n - k')!} \geq (n - 2)(n - 3)$. But

$$r' - 1 + n + (r' - 1)k' - F(r') = (r' - 1)(k' - 1) + n - 2$$

$$\leq (n - 4)(n - 2) + n - 2 = (n - 2)(n - 3).$$

We used the inequalities $r' - 1 \leq n - k' \leq n - 4$ and $k' - 1 \leq n - 2$, so equality holds if and only if equality holds in both these inequalities. However, the first inequality being equality implies that $k' = 4$, and then, the second being equality implies $n = 5$. Then, going back to the first inequality, we have that $r' = n - 3 = 2$, which contradicts our supposition that
$r' \geq 4$. Thus, strict inequality holds and since the choice of $i$ was arbitrary, there is an edge between the large component of $H_i - T_i$ and $H_l - T_l$ for every admissible $i$, and hence, all these large components are part of one component in $S_{n,k'} - T$.

Next, we show that the small components have at most $r' - 1$ vertices in total, so suppose the contrary. Note that in the second case of the induction in the proof of Theorem 4.1, we did not use the definition of $F(x)$ until after we derived the inequality $\sum_{i=1}^{j} F(p_i) - F(r') + r' \geq (j - 1)(n - k') + j$. Thus, it still holds. Using the new definition of $F$ and the fact that $\sum_{i=1}^{j} p_i = r'$, we find that $r' - j \geq (j - 1)(n - k')$. Note that earlier, we also did not use the definition of $F(x)$ in order to derive $j \geq 2$, so that still holds. Thus, since $r' \leq n - k' + 1$, we have $(j - 1)(n - k') \geq (n - k') \geq r' - 1 > r' - j$, a contradiction. Thus, at most $r' - 1$ vertices are in the small components, completing our induction. \(\square\)

It remains to show that in the case when $r \leq n - k + 1$, the above proven bound is best possible. Consider the $r$ vertices corresponding to the permutations $[1, n - k + 2, n - k + 3, \cdots, n]$, $[2, n - k + 2, n - k + 3, \cdots, n]$, $\cdots$, $[r, n - k + 2, n - k + 3, \cdots, n]$, which exist since $r \leq n - k + 1$. The vertices obtained by applying adjacency rule (1) by applying the transposition $(1, j)$ for $3 \leq j \leq k$ to each of these $r$ permutations give $k - 1$ distinct permutations per vertex, for a total of $r(k - 1)$ permutations. The vertices obtained by adjacency rule (2) give $n - k - r + 1$ choices for the first element in the permutation, and so $n - k - r + 1$ permutations. Thus, the total is $n + (r - 1)k - 2r + 1$ vertices in the union of the neighbor sets of the vertices in consideration. Since the deletion of this union of the neighbor sets will disconnect $r$ vertices, it follows that $n + (r - 1)k - 2r$ is a best possible bound, as desired.

5. Applications to other types of connectivity

The results of this paper immediately resolve many other connectivity issues for $(n,k)$-stars. An $m$-regular graph is known as tightly super-connected if the deletion of any minimal disconnecting set results in two components, one of which is a singleton. Super-connectivity
was examined previously for certain other graphs in [3, 7, 11]; the following result examines it for $S_{n,k}$.

**Theorem 5.1.** $S_{n,k}$ is tightly super-connected when $n > k > 2$.

*Proof.* By Theorem 3.1, the minimum disconnecting set has size $n - 1$. However, since $n - 1 < n + k - 4$, it follows by Theorem 3.2 that deletion of this many vertices either results in a connected graph or a large component and a singleton, as desired. □

In fact, it is easy to see that $S_{n,2}$ is not tightly super-connected. We refer readers to [5, 16, 17] for additional history behind the following two types of connectivity in other graphs.

1. **$R^m$-vertex-connectivity:** Let $G$ be a graph. Then, $T \subseteq V(G)$ is called an $R^m$-vertex-cut if $G - T$ is disconnected and each vertex in $V(G) - T$ has at least $m$ neighbors in $G - T$. The size of the smallest $R^m$-vertex-cut is called the $R^m$-vertex-connectivity, denoted $\kappa^m(G)$. We have the following:

**Theorem 5.2.** For $n > k \geq 3$, $\kappa^m(S_{n,k}) = n + mk - 2m - 1$ when $m \leq n - k$.

*Proof.* By Theorem 4.2, the deletion of at most $n + mk - 2(m + 1)$ vertices cannot disconnect more than $m$ vertices, so the small component cannot have each vertex with degree at least $m$ as required. Thus, it suffices to show that there exists an $R^m$-vertex-cut of size $n + mk - 2m - 1$. However, consider the vertex cut given after the proof of Theorem 4.2. Clearly, the small component is isomorphic to $K_{m+1}$ and so all vertices have degree exactly $m$, fitting the requirements. Furthermore, it is easy to see that each vertex still remaining in the large component has at most one neighbor deleted, and thus, has degree $n - 2 > n - k \geq m$, as required. □

2. **Cyclic vertex-connectivity:** Let $G$ be a graph. Then $T \subseteq V(G)$ is called a cyclic vertex-cut if $G - T$ is disconnected and at least two components in $G - T$ contain a cycle. The cyclic vertex-connectivity, denoted $\kappa_c(G)$, is the size of a smallest cyclic vertex-cut. It is known that $\kappa_c(S_{n,n-1}) = 6(n - 3)$. For non-star graphs, we have the following:
**Theorem 5.3.** For $n - 1 > k \geq 3$, $\kappa_c(S_{n,k}) = n + 2k - 5$.

*Proof.* By Theorem 3.3, we have that if fewer than $n + 2k - 5$ vertices are deleted, the small component has at most 2 vertices and cannot have a cycle. The construction after the proof of Theorem 3.3 shows that deletion of $n + 2k - 5$ vertices can disconnect a $K_3$, which is a cycle, and clearly the large component has a cycle, as desired. \[\Box\]

6. Conclusions

In this paper, we examined the maximum number of vertices that could be deleted from an $(n,k)$-star graph, a generalization of the star graph, to still guarantee that the resulting graph is either connected or has a large component and small components with at most $r - 1$ vertices in total. First, we proved bounds on the connectivity of $(n,k)$-star graphs when small numbers of vertices were deleted in Theorems 3.2 and 3.3. These bounds were extended to any linear number of deletions to give both asymptotic and precise results in Theorems 4.1 and 4.2. The expressions in the results for larger $r$ took the form of $n + (r - 1)k - f(r)$ for some function of $r$, which approaches $r(n - 1) - f(r)$ as $k$ approaches $n - 1$. Deletion of $r(n - 1)$ vertices can disconnect $r$ vertices from any $(n - 1)$-regular graph, and thus, a number in the form $r(n - 1) - f(r)$ for the number of deletions in which at most $r - 1$ vertices are disconnected is a very sharp bound as $f(r)$ is small. Thus, it can be concluded that $(n,k)$-star graphs with values of $k$ close to $n$ are most resistant to vertex deletions. The value for $f(r)$ was proven to be linear in $r$ in the case when $n - k + 1 \geq r$ by Theorem 4.2. In other cases, $f(r)$ was proven to be no more than quadratic by Theorem 4.1.

The problem of the $n$-dimensional star graph having $n!$ vertices is solved with $(n,k)$-stars, which have $\frac{n!}{(n-k)!}$ vertices. The star graphs with between 1000 and 200,000 vertices are only the star graphs for $n = 7$ and 8. If we allow $(n,k)$-stars and bound $k \geq n - 3$ so that by the above, the structural properties are still good, we still have, in addition to the star graphs, the options $S_{7,5}$, $S_{8,6}$, $S_{8,5}$, $S_{9,7}$, and $S_{9,6}$. Thus, this paper has resolved the fundamental problem with star graphs while still retaining the desirable structural properties.
Further research can be done on when significantly more vertices are deleted. Theorem 4.1 becomes weak as \( r \) approaches and gets larger than \( n \) and Theorem 4.2 does not cover these cases. Thus, the structural properties of \((n, k)\)-stars are not resolved when more than linearly many vertices are deleted; for example, it remains to study when quadratically many, cubically many, or any polynomial number vertices are deleted. As \((n, k)\)-stars can have a factorial number of vertices, further study can also resolve the case of even larger vertex deletions.

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MATCHING PRECLUSION AND CONDITIONAL MATCHING PRECLUSION FOR AUGMENTED CUBES

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The matching preclusion number of a graph is the minimum number of edges whose deletion results in a graph that has neither perfect matchings nor almost-perfect matchings. For many interconnection networks, the optimal sets are precisely those incident to a single vertex. Recently, the conditional matching preclusion number of a graph was introduced to look for obstruction sets beyond those incident to a single vertex. It is defined to be the minimum number of edges whose deletion results in a graph with no isolated vertices that has neither perfect matchings nor almost-perfect matchings. In this paper, we find this number and classify all optimal sets for the augmented cubes, a class of networks designed as an improvement of the hypercubes.

Keywords: Interconnection network, perfect matching, augmented cube.

1. Introduction and Preliminaries

A perfect matching in a graph is a set of edges such that every vertex is incident with exactly one edge in this set. An almost-perfect matching in a graph is a set of

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edges such that every vertex except one is incident with exactly one edge in this set, and the exceptional vertex is incident to none. So if a graph has a perfect matching, then it has an even number of vertices; if a graph has an almost-perfect matching, then it has an odd number of vertices. The matching preclusion number of a graph \( G \), denoted by \( \text{mp}(G) \), is the minimum number of edges whose deletion leaves the resulting graph without a perfect matching or almost-perfect matching. Any such optimal set is called an optimal matching preclusion set. We define \( \text{mp}(G) = 0 \) if \( G \) has neither a perfect matching nor an almost-perfect matching. This concept of matching preclusion was introduced by Brigham et al.\(^1\) and further studied in other papers.\(^2,4,13\) They introduced this concept as a measure of robustness in the event of edge failure in interconnection networks, as well as a theoretical connection to conditional connectivity, “changing and unchanging of invariants” and extremal graph theory. We refer the readers to Brigham et al.\(^1\) for details and additional references. We use standard notations and terminology as in West.\(^16\)

Useful distributed processor architectures offer the advantage of improved connectivity and reliability. An important component of such a distributed system is the system topology, which defines the inter-processor communication architecture. In certain applications every vertex requires a special partner at any given time and the matching preclusion number measures the robustness of this requirement in the event of link failures as indicated in Brigham et al.\(^1\) Hence in these interconnection networks, it is desirable to have the property that the only optimal matching preclusion sets are those whose elements are incident to a single vertex.

**Proposition 1.1.** Let \( G \) be a graph with an even number of vertices. Then \( \text{mp}(G) \leq \delta(G) \), where \( \delta(G) \) is the minimum degree of \( G \).

**Proof.** Deleting all edges incident to a single vertex will give a graph with no perfect matchings and the result follows. \(\square\)

We call an optimal solution of the form given in the proof of Proposition 1.1 a trivial optimal matching preclusion set. As mentioned earlier, it is desirable for an interconnection network to have only trivial optimal matching preclusion sets. In the event of random failure, it is unlikely that every edge incident to a particular vertex will be faulty. Thus, it is natural to ask what the next obstruction sets are for a graph with link failure subject to the condition that the faulty graph has no isolated vertices. This motivates the definition given in Cheng et al.\(^3\) and further studied by others.\(^5,14\) The conditional matching preclusion number of a graph \( G \), denoted by \( \text{mp}_1(G) \), is the minimum number of edges whose deletion leaves the resulting graph with no isolated vertices and without a perfect matching or almost-perfect matching. Any such optimal set is called an optimal conditional matching preclusion set. We define \( \text{mp}_1(G) = 0 \) if \( G \) has neither a perfect matching nor an almost-perfect matching. We will leave \( \text{mp}_1(G) \) undefined if a conditional matching preclusion set does not exist, that is, we cannot delete edges to satisfy both conditions in
the definition. Therefore, the question is: by deleting edges, what are the basic obstructions to a perfect matching or an almost-perfect matching in the resulting graph if no isolated vertices are created? In Proposition 1.1, we see that without the condition of no isolated vertices, an isolated vertex will be the basic obstruction and so deleting all edges incident to \( G \) will produce a trivial matching preclusion set. Now for a resulting graph with no isolated vertices, a basic obstruction to a perfect matching will be the existence of a path \( u - w - v \) where the degree of \( u \) and the degree of \( v \) are 1. So to produce such an obstruction set, one can pick any path \( u - w - v \) in the original graph and delete all the edges incident to either \( u \) or \( v \) but not the edges \((u, w)\) and \((w, v)\). We define

\[
\nu_e(G) = \min \{d_G(u) + d_G(v) - 2 - y_G(u,v) : u \text{ and } v \text{ are ends of a path of length 2}\}
\]

where \( d_G(\cdot) \) is the degree function and \( y_G(u,v) = 1 \) if \( u \) and \( v \) are adjacent and 0 otherwise. (We will suppress \( G \) and simply write \( d \) and \( y \) if it is clear from the context.) So mirroring Proposition 1.1, we have the following easy result which follows directly from the definition of \( \nu_e(G) \).

**Proposition 1.2.** Let \( G \) be a graph with an even number of vertices. Suppose every vertex in \( G \) has degree at least three. Then

\[
mp_1(G) \leq \nu_e(G).
\]

We note that the condition “\( \delta(G) \geq 3 \)” ensures that the resulting graph (after edges have been deleted) has no isolated vertices. Moreover, this condition is not strictly necessary if we are willing to exclude certain exceptions such as a 4-cycle. For our purposes, Proposition 1.2 suffices.

We call an optimal solution of the form induced by \( \nu_e \) a trivial optimal conditional matching preclusion set. As mentioned earlier, the matching preclusion number measures the robustness of this requirement in the event of link failures, so it is desirable to have the property that the only optimal matching preclusion sets are the trivial ones. Similarly, it is desirable to have the property that the only optimal conditional matching preclusion sets are the trivial ones as well. Cheng et al.\(^3\) introduced this concept and considered the conditional matching preclusion problem for a number of basic networks including the hypercubes and they were proven to have this desired property. The augmented cubes are designed to be superior to the hypercubes.\(^6\) Not only do they retain many favorable properties of the hypercubes, they have some important embedding properties that the hypercubes do not have. So it is natural to ask whether the augmented cubes measure up under these parameters of matching preclusion and conditional matching preclusion. Some recent papers\(^7\)–\(^12\),\(^17\) on the augmented cubes studied fault Hamiltonicity and cycle embedding among many others.

An \( n \)-dimensional augmented cube, denoted by \( AQ_n \), has \( 2^n \) vertices, each labeled by an \( n \)-bit binary string \( u_1 u_2 \ldots u_n \). \( AQ_1 \) is defined as two vertices, labeled 0 and 1,
that are adjacent. \(AQ_n\) can be recursively defined by taking two copies of \((n-1)\)-dimensional augmented cubes. We add the digit 0 to the beginning of the binary strings of all vertices in one augmented cube, and add the digit 1 to the beginning of the vertices of the second. Finally, we construct an edge between vertices \(u\) and \(v\) in different \(AQ_{n-1}\) if and only if, in their bitstring representations:

\[(1) \quad u_i = v_i \text{ for every } i > 1.\]
\[
\text{In this case, we call the edge }(u,v)\text{ a cross edge and denote } v = u^h \text{ and } u = v^h, \text{ or}
\]
\[(2) \quad u_i \neq v_i \text{ for every } i \geq 1.\]
\[
\text{We call the edge }(u,v)\text{ a complement edge and denote } v = u^c \text{ and } u = v^c.
\]

Throughout this paper we let \(S_n\) and \(C_n\), or simply \(S\) and \(C\) if it is clear from the context, denote the set of cross edges and the set of complement edges in \(AQ_n\), respectively. The augmented cube and its properties were defined and studied by Choudum and Sunitha.\(^6\) One important property is that it is vertex-transitive and it has a recursive property by the definition. Observe that the graph \(AQ_n\) is \((2^n-1)\)-regular, and also \(AQ_n\) is constructed from two copies \(AQ_{n-1}\) connected by a set of cross edges and complement edges. It follows from the definition that for two neighboring vertices \(a\) and \(b\), \(a^h\) and \(b^h\) will be neighbors, as well as \(a^c\) and \(b^c\). In \(AQ_n\), each of the set of cross edges and the set of complement edges form perfect matchings. Suppose we denote the subgraph of \(AQ_n\) induced by the vertices with a leading 0 by \(AQ^0_{k-1}\) and the subgraph of \(AQ_n\) induced by the vertices with a leading 1 by \(AQ^1_{k-1}\). Then each of \(AQ^0_{k-1}\) and \(AQ^1_{k-1}\) is isomorphic to \(AQ_{k-1}\). Figure 1 gives some examples of the augmented cubes. (Throughout the paper, blue, straight edges represent cross edges, red curved edges represent complement edges, and black edges represent neither.)

In this paper, we proved that, like the hypercubes, the augmented cubes have favorable properties with respect to matching preclusion and conditional matching preclusion. We find these numbers and classify all the optimal solutions. Basically, all optimal solutions are trivial. This further solidifies the competitiveness of the augmented cubes as interconnection networks.

We would now comment on the proof that we use in this paper. The idea is to use induction. Here checking the base cases requires a computer program. Although more is known for the matching preclusion problem, much less is known for the conditional matching preclusion problem. Not counting the complete and the complete bipartite graphs which are not suitable as interconnection networks, the main classes of graphs for which the matching preclusion problem is solved are the hypercubes, the star graphs and their generalization, the HL-graphs, and the alternating group graphs. Most of the proofs rely on existing results on fault Hamiltonicity. For the conditional matching preclusion problem, the only complete results published on usable interconnection networks are the hypercubes and the star graphs. (The result by Park and Son\(^14\) does not include the classification of optimal solutions.) Again, results on fault Hamiltonicity were used in the proof for the star graphs.
However, these fault Hamiltonicity results are in a sense “too strong.” In this paper, no advanced results are used and the proofs are self-contained. We remark that for regular bipartite graphs, determining the matching preclusion number is easy as the edges of an $r$-regular bipartite graphs can be partitioned into $r$ edge-disjoint perfect matchings. In addition, the matching preclusion number can also be found easily for regular non-bipartite graphs provided that the graph has high connectivity based on a result of Plesník.\(^{15}\) (Although this result can be used to establish the matching preclusion number for $AQ_n$, it is easier to give a self-contained proof via Proposition 2.1.) However, we are not aware of any published results in determining the conditional matching preclusion number, classifying optimal matching preclusion sets and classifying optimal conditional matching preclusion sets for general graphs even if additional conditions are included. It is also unknown whether there is a polynomial time algorithm to compute these numbers.

2. Matching Preclusion

**Proposition 2.1.** Let $n \geq 1$. Then the edges of $AQ_n$ can be partitioned into $2n - 1$ edge-disjoint perfect matchings.

**Proof.** It is easy to see that the claim is true for $AQ_1, AQ_2, AQ_3$. Now the result follows from an induction argument, and the fact that each of $S_n$ and $C_n$ gives a perfect matching of $AQ_n$. ($2n - 3$ perfect matchings by induction on $AQ_{n-1}$ and $AQ_{n-1}^1$ together with $S_n$ and $C_n$ give the desired set of edge-disjoint perfect matchings of $AQ_n$.)

**Proposition 2.2.** Let $n \geq 2$. Then the subgraph of $AQ_n$ induced by $S$ and $C$ spans $AQ_n$. Moreover, it is a 2-regular graph with each component being a 4-cycle having exactly two cross edges and two complement edges, and the cross edges and complement edges appear in an alternating manner.
Proof. The result follows directly from the observation that $1u - 0u - 1\nu - 0\nu - 1u$ is a 4-cycle.

We remark that Proposition 2.2 immediately implies that to destroy every perfect matching in $AQ_n$, we need to delete at least one edge in $S$ and at least one edge in $C$. Moreover, we must have a pair of such edges (one from each of $S$ and $C$) coming from the same 4-cycle which are incident to the same vertex.

Theorem 2.1. Let $n \geq 1$. Then $mp(AQ_n) = 2n - 1$. If $n \geq 3$, then every optimal matching preclusion set is trivial.

Proof. The first statement follows directly from Proposition 2.1. We will now classify all the optimal matching preclusion sets. One can easily check that the statement is correct for $AQ_3$. Assume that the classification statement is true for some $n = k - 1 \geq 3$. Define $AQ_{k-1}^1$ and $AQ_{k-1}^0$ as usual. Let $F$ be an optimal matching preclusion set of $AQ_k$, so $|F| = 2k - 1$. Let $F_1 = F \cap E(AQ_{k-1}^1)$, $F_0 = F \cap E(AQ_{k-1}^0)$, $F_s = S \cap F$ and $F_c = C \cap F$. Since $S$ and $C$ are perfect matchings in $AQ_k$, $|F_s|, |F_c| \geq 1$.

So $|F_1| + |F_0| \leq 2k - 3$. Since $AQ_k - F$ has no perfect matchings, at least one of $AQ_{k-1}^1 - F_1$ and $AQ_{k-1}^0 - F_0$ has no perfect matchings. Since $mp(AQ_{k-1}) = 2k - 3$, either $|F_1| = 2k - 3$ or $|F_0| = 2k - 3$. Without loss of generality, assume $|F_1| = 2k - 3$ and hence $|F_s| = |F_c| = 1$ and $|F_0| = 0$. It now follows from the induction hypothesis that the edges in $F_1$ are incident to the same vertex $v$ in $AQ_{k-1}^1$. It follows from Proposition 2.2 that the two edges in $F_s \cup F_c$ are incident to some vertex $u$. (If the edges are in different 4-cycles, then $(S \cup C) - (F_s \cup F_c)$ still contains a perfect matching of $AQ_k$.) If $u = v$, then we are done. So either $(v, v^h)$ or $(v, v^c)$ is not in $F$.

The two cases are similar. So we assume $(v, v^h)$ is not in $F$. Let the unique element of $F_s$ be $(a, a^h)$ with $a$ in $AQ_{k-1}^1$. (This means $a$ is either $a$ or $a^h$.) By assumption $a \neq v$. So $a$ has some neighbor $c$ in $AQ_{k-1}^1$ with $(a, c) \notin F_1$ since $a$ has at least five neighbors in $AQ_{k-1}^1$ and at least one of them is not $v$. Now notice that $a^h$ is adjacent to $c^h$ in $AQ_{k-1}^0$. Let $M = (S - \{(a, a^h), (c, c^h)\}) \cup \{(a, c), (a^h, v^c)\}$. Then $M$ is a perfect matching in $AQ_k - F$, a contradiction. This completes the proof.

We note that Theorem 2.1 is the best possible as $AQ_2$, isomorphic to $K_4$ (a complete graph on four vertices), contains a non-trivial optimal matching preclusion set. Indeed, one can delete the edges of a triangle, leaving a $K_{1,3}$ (a complete bipartite graph with the two bipartition sets having size one and three) with no perfect matchings. However, this is the only exception.

3. Conditional Matching Preclusion

We now study the conditional matching preclusion problem for the augmented cubes. Since $AQ_n$ is $(2n - 1)$-regular and it contains triangles, $\nu_c(AQ_n) = 4n - 5$. So a trivial conditional matching preclusion set is of the form given in Figure 2, that is, take three pairwise adjacent vertices $a, b, c$, delete all edges incident to $a$ but not $(a, b)$,
and delete all edges incident to \( c \) but not \((c, b)\). We start with a couple of useful propositions.

Proposition 3.1. Let \( n \geq 2 \). If \( u \) and \( v \) are two distinct vertices in \( AQ_n \), then \( AQ_n - \{u, v\} \) has a perfect matching.

Proof. We proceed by induction on \( n \). A simple hand check confirms our base case \( AQ_2 \). Assume the statement is true for some \( n = k - 1 \geq 2 \). Define the \( AQ^i_{k-1}'s \) as usual. There are two cases. If both \( u \) and \( v \) are in the same \( AQ^i_{k-1} \), then we simply use our induction hypothesis and we are done. If \( u \) is in \( AQ^1_{k-1} \) and \( v \) is in \( AQ^0_{k-1} \), then find two cross edges covering \( u \) and \( v \) together with an additional vertex in \( AQ^1_{k-1} \) and an additional vertex in \( AQ^0_{k-1} \). (If \( v \neq u^h \), then pick \((u, u^h)\) and \((v^h, v)\); otherwise, pick \((u, u^h)\) and any other cross edge.) Now apply the induction hypothesis twice to complete the proof.

Proposition 3.2. Let \( n \geq 2 \). If \( e \) and \( f \) are two independent edges in \( AQ_n \), then there exists a perfect matching of \( AQ_n \) containing \( e \) and \( f \).

Proof. We will prove by induction on \( n \), with base case \( AQ_2 \) being trivial. Assume the statement is true for some \( n = k - 1 \geq 2 \). Define the \( AQ^i_{k-1}'s \) as usual. If both edges are in the same \( AQ^i_{k-1} \) then we are done by our induction hypothesis. If they are in separate \( AQ^i_{k-1} \), then we can simply use another independent edge in each \( AQ^i_{k-1} \) and we are again done by our induction hypothesis. If both edges are cross edges or both are complement edges then our perfect matching is either \( S \) or \( C \), respectively. If \( e \) is a cross edge and \( f = (a, b) \), is in an \( AQ^i_{k-1} \), then our desired perfect matching is \( \{(a, b), (a^h, b^h)\} \cup (S - \{(a, a^h), (b, b^h)\}) \). (Note that \( e \notin \{(a, a^h), (b, b^h)\} \) as \( e \) and \( f \) are independent.) If \( e \) is a complement edge and \( f = (a, b) \), the proof is similar. Finally, suppose \( e = (a, b) \) is a cross edge and \( f = (c, d) \) is a complement edge where \( a \) and \( c \) are in \( AQ^1_{k-1} \). Then use \( e \) and \( f \), and apply Proposition 3.1 to each of \( AQ^1_{k-1} - \{a, c\} \) and \( AQ^0_{k-1} - \{b, d\} \).

Fig. 2. A trivial conditional matching preclusion in \( AQ_n \).
represent cross edges, red curved edges represent complement edges, and black edges represent neither. Now, edges that are dotted represent parts of a perfect matching. Grayed out edges or those with red crosses represent edges that were removed and cannot be used in a perfect matching. Finally, when a vertex is incident to a number of grayed out edges, but the other vertices of these edges are not drawn, we assume that this vertex is isolated from all neighbors other than those that are drawn. Small circles represent vertices. Filled black vertices represent those that have been matched with some other vertex, while unfilled white vertices have not yet been matched.

Our goal is to prove that $mp_1(AQ_n) = 4n - 5$ and every optimal conditional matching preclusion set is trivial. Since $AQ_2$ has a non-trivial matching preclusion set, we consider $n \geq 3$. Unfortunately, the claim is not true for $n = 3$. Indeed, $mp_1(AQ_3) = 6$ and not 7, as expected. Since $mp(AQ_3) = 5$ and all optimal matching preclusion sets are trivial, $mp_1(AQ_3) \geq 6$ and Figure 3 shows that $mp_1(AQ_3) = 6$. (The Tutte set for the graph in Figure 3 is $\{3, 6, 8\}$.) We note that this conditional matching preclusion set contains a triangle, and this triangle is in a $K_4$ on the right. This “bad” non-trivial matching preclusion set percolates to $AQ_3$ and $AQ_3$ is not large enough to dilute this bad case. Fortunately $AQ_n$ with $n \geq 4$ is large enough.

The next result can be established by brute force.

**Lemma 3.1.** $mp_1(AQ_3) = 6$ and the set of shaded edges in Figure 3 is the unique optimal conditional matching preclusion set, up to automorphisms. Moreover, if $F$ is a conditional matching preclusion set with $|F| = 7$, then $F$ is either the trivial conditional matching preclusion set, $F$ contains the set of shaded edges in Figure 3, or $F$ is the set of shaded edges in Figure 4, Figure 5 or Figure 6, up to automorphisms.

We note that Lemma 3.1 can be checked via brute force by hand exploiting symmetries, without the aid of a computer program. It will be used to establish the result for $AQ_4$, again, by brute force but this time with a computer program.

**Lemma 3.2.** $mp_1(AQ_4) = 11$. Moreover, every optimal conditional matching preclusion set is trivial.
Fig. 4. A conditional matching preclusion set of $AQ_3$ of size 7.

Fig. 5. A conditional matching preclusion set of $AQ_3$ of size 7.

Fig. 6. A conditional matching preclusion set of $AQ_3$ of size 7.
Proof. Let \( F \) be a set of edges of size 11 such that \( AQ_4 - F \) has no isolated vertices. Since \( AQ_4 \) has 56 edges and we need to delete 11 of them, it is infeasible to use a computer program naively and check all possible cases. Instead, we use a combination of a theoretical approach and a computational approach. Define \( AQ_3 \)'s, \( F_1, F_0, F_s, F_c \) as usual. The proof involves two steps. The first step is to justify the following claim: If \( |F_1| \geq 6 \) and \( F_1 \) contains the conditional matching preclusion set for \( AQ_3 \) given in Figure 3, then \( AQ_4 - F \) has a perfect matching; if \( |F_1| \geq 7 \) and \( F_1 \) contains the conditional matching preclusion set for \( AQ_3 \) given in Figure 4, Figure 5 or Figure 6, then \( AQ_4 - F \) has a perfect matching. Although Step 1 can be verified by hand taking advantage of the symmetries, it is more efficient to use a computer program. Indeed the computer verified the claim in about two hours (clock time). The next step is to assume \( F \) is not covered in Step 1. Then we consider whether \( AQ_3^1 - F_1 \) and \( AQ_3^0 - F_0 \) have isolated vertices. Indeed the analysis is essentially an adaptation of the proof given in Theorem 3.1 when the induction step is given. The proof is given in the appendix.

We are now ready for the main result.

Theorem 3.1. Let \( n \geq 4 \). Then \( mp_1(AQ_n) = 4n - 5 \). Moreover, every optimal conditional matching preclusion set is trivial.

Proof. We will give the outline of the proof before we go into the details. First, we note that the two statements can be proved together by proving the following: Let \( F \) be a set of edges in \( AQ_n \) where \( |F| \leq 4n - 5 \) and that \( AQ_n - F \) has no isolated vertices. Then either \( AQ_n - F \) has a perfect matching or \( F \) is a trivial conditional matching preclusion set (so \( |F| = 4n - 5 \)). It is this statement that we will prove using induction. The case \( n = 4 \) is covered by Lemma 3.2, so assume the statement is true for some \( n = k + 1 \geq 4 \). Define \( AQ_{k-1}^1 \)'s, \( F_1, F_0, F_s, F_c \) as usual. The two main cases are whether \( AQ_{k-1}^1 - F_1 \) and \( AQ_{k-1}^0 - F_0 \) have isolated vertices. If \( AQ_k - F \) has a perfect matching, then we are done. Henceforth, we may assume \( AQ_k - F \) has no perfect matchings. So \( |F_s|, |F_c| \geq 1 \), so \( |F_1| + |F_0| \leq 4k - 7 \). It follows from Proposition 2.2 that there is a vertex incident to a cross edge in \( F_s \) and a complement edge in \( F_c \).

Case 1: \( AQ_{k-1}^1 - F_1 \) and \( AQ_{k-1}^0 - F_0 \) have no isolated vertices. Without loss of generality, we may assume \( |F_1| \geq |F_0| \). Since there are no isolated vertices in either \( AQ_{k-1}^1 \), if \( |F_1| \leq 4k - 10 = 4(k - 1) - 6 \), then \( |F_0| \leq 4(k - 1) - 6 \), and hence each \( AQ_{k-1}^1 - F_1 \) and \( AQ_{k-1}^0 - F_0 \) has a perfect matching. This implies \( AQ_k - F \) has a perfect matching, a contradiction. Therefore \( |F_1| \) is \( 4k - 9 \), \( 4k - 8 \) or \( 4k - 7 \). So we consider three subcases.

Subcase 1: \( |F_1| = 4k - 9 \). Then \( |F_0| \leq 2 \) and hence \( AQ_{k-1}^0 - F_0 \) has a perfect matching by Theorem 2.1 as \( 2(k - 1) - 1 > 2 \). Thus \( AQ_{k-1}^0 - F_1 \) must have no perfect matchings. Therefore, by our induction hypothesis, \( F_1 \) is a trivial conditional matching preclusion set of \( AQ_{k-1}^1 \). So there are pairwise adjacent vertices \( a, b, c \) in
AQ_{k-1}^1 such that all edges adjacent to a and b are in F except for (a, c) and (b, c). If all cross or complement edges incident to a and b are in F, then we are done as F is a trivial conditional matching preclusion set in AQ_k. Therefore, assume without loss of generality that the cross edge incident to a is not in F. Since |F_s| ≤ 3 and a has 2(k - 1) - 1 > 3 neighbors, a has a neighbor d such that (d, d^h) ̸∈ F_s. Let $F'_1 = F_1 - \{(a, d)\}$. Then |$F'_1$| = 4k - 10 < 4(k - 1) - 5 and AQ_{k-1}^1 - F'_1 has no isolated vertices. So AQ_{k-1}^1 - F'_1 has a perfect matching $M'_1$ by the induction hypothesis. Clearly (a, d) ∈ $M'_1$. The idea is to extend $M'_1 - \{(a, d)\}$ to a perfect matching of AQ_k by adding (a, a^b) and (d, d^b) to it together with a perfect matching of AQ_{k-1}^0 - \{(a^b, d^h)\}. Now consider AQ_{k-1}^0 - F_0 has a perfect matching $M_0'$ and clearly (a^b, d^b) ∈ $M_0'$. Now (M'_1 - \{(a, d)\}) ∪ (M_0' - \{(a^b, d^h)\}) ∪ \{(a, a^b), (d, d^b)\} is a perfect matching of AQ_k - F, a contradiction.

Subcase 2: |$F_1$| = 4k - 8. Then |$F_0$| ≤ 1 and hence AQ_{k-1}^0 - F_0 has a perfect matching by Theorem 2.1 as 2(k - 1) - 1 > 1. Now since |F_s|, |F_c|, |F_0| ≤ 1 and |$F_s$|, |$F_c$| ≥ 1, either |$F_s$| = 1 or |$F_c$| = 1. Without loss of generality, assume |$F_s$| = 1 and its unique cross edge is (a, a^h) where a is in AQ_{k-1}^1. Since AQ_{k-1}^1 - F_1 has no isolated vertices, a has at least one neighbor in AQ_{k-1}^1 - F_1. If a has more than one neighbor in AQ_{k-1}^1 - F_1, we claim that there is a perfect matching in AQ_{k-1}^1 - F_1. This is because if a has two such neighbors y and c, we may assume (a^h, y^h) ̸∈ F_0 as |$F_0$| ≤ 1. Hence ($S - \{(a^h, y^h)\}$) ∪ \{(a, y), (a^h, y^h)\} is a perfect matching of AQ_k - F, a contradiction. Hence, a must have exactly one neighbor in AQ_{k-1}^1 - F_1, say y, as it has no isolated vertices. Indeed, the preceding argument is still valid if a has only one neighbor y in AQ_{k-1}^1 - F_1 as long as (a^h, y^h) ̸∈ F_0. So we may assume (a^h, y^h) ∈ F_0. This implies |$F_s$|, |$F_c$|, |$F_0$| = 1.

We have now found 2k - 4 elements of F_1, namely, those incident to a, except (a, y), in AQ_{k-1}^1. Let (b, c) be any other element in F_1, that is, it is not incident to a. Let $F'_1 = F_1 - \{(b, c)\}$. Then |$F'_1$| = 4k - 9 = 4(k - 1) - 5. So by the induction hypothesis, either AQ_{k-1}^1 - F'_1 has a perfect matching $M'_1$ or $F'_1$ is a trivial conditional matching preclusion set in AQ_{k-1}^1. Suppose such a $M'_1$ exists. Then (b, c) ∈ $M'_1$. The idea is to extend ($M'_1 - \{(b, c)\}$) ∪ \{(b, b^h), (c, c^h)\} to a perfect matching of AQ_k - F. Now let $F''_0$ be all the edges in AQ_{k-1}^0 that are incident to b^h except (b^h, c^h). Then let $F_0 = F_0 ∪ F''_0 = \{(a^b, y^h)\} ∪ F''_0$. So |$F_0$| = |2(k - 1) - 2 + 1| = 2k - 3 < 4(k - 1) - 5 since k ≥ 5. Clearly AQ_{k-1}^0 - F_0 has no isolated vertices as 2(k - 1) - 1 > 2. Then by the induction hypothesis, AQ_{k-1}^0 - F'_1 has a perfect matching $M_0'$ and clearly (b^h, c^h) ∈ $M_0'$. Now, ($M'_1 - \{(b, c)\}$) ∪ \{(b, b^h), (c, c^h)\} ∪ (M_0' - \{(b^h, c^h)\}) is a perfect matching of AQ_k - F, a contradiction. Now suppose $F'_1$ is a trivial conditional matching preclusion set in AQ_{k-1}^1. Then a and some other vertex d must have been isolated from all vertices but one common neighbor, namely, y. Note that b, c ̸= d,
otherwise we would have originally isolated $d$. Therefore, we pick any $(d, e) \in F_1$ where $e \neq a$. (Such $e$ exists as $2(k - 1) - 1 > 1$). Now consider $F''_1 = F_1 - \{(d, e)\}$. Then $F''_1$ is not a trivial conditional matching preclusion set in $AQ^1_{k-1}$, so $AQ^1_{k-1} - F''_1$ has a perfect matching $M''_1$ as it has no isolated vertices. We note that $(d, e) \in M''_1$ as $AQ^1_{k-1} - F_1$ has no perfect matchings. Now, proceed as before to obtain a perfect matching $M''_1$ in $(AQ^0_{k-1} - F_0) - \{(d, e)\}$. So $M''_1 - \{(d, e)\} \cup \{(d, d^0), (e, e^0)\} \cup M''_1$ is a perfect matching of $AQ_k - F$, a contradiction.

Subcase 3: $|F_1| = 4k - 7$. Then $|F_0| = 0$ and $|F_s| = |F_c| = 1$. So $AQ^0_{k-1} - F_0 = AQ^0_{k-1}$. Consider the unique cross edge in $F$, say, $(a, a^h)$ with vertex $a$ in $AQ^1_{k-1}$. By assumption, $a$ is not an isolated vertex in $AQ^1_{k-1} - F_1$. Therefore, there is some vertex $e$ in $AQ^1_{k-1} - F_1$ that is adjacent to $a$ in $AQ^1_{k-1} - F_1$. Then $(S - \{(a, a^h), (c, e^h)\}) \cup \{(a, c), (a^h, c^h)\}$ is a perfect matching of $AQ_k - F$, a contradiction.

Case 2: $AQ^1_{k-1} - F_1$ and/or $AQ^0_{k-1} - F_0$ contain isolated vertices. Without loss of generality, $AQ^1_{k-1} - F_1$ contains isolated vertices. Then $|F_1| \geq 2k - 3$. So $|F_0| \leq 4k - 5 - |F_1| - |F_s| - |F_c| \leq 4k - 5 - (2k - 3) - 1 - 1 = 2k - 4$. So $AQ^0_{k-1} - F_0$ has no isolated vertices. Since $|F_s|, |F_c| \geq 1, |F_1| \leq 4k - 7$. Hence if $AQ^1_{k-1} - F_1$ has two isolated vertices $u$ and $v$, then they must be adjacent and that $|F_1| = 4k - 7$. This implies $|F_s| = |F_c| = 1$ and $|F_0| = 0$. Let $(a, a^h)$ be the unique element in $F_s$ where $a$ is in $AQ^1_{k-1}$. Suppose $a \notin \{u, v\}$. Then pick a neighbor of $a$, say $b$, that is neither $u$ nor $v$. (Such a $b$ exists as $2k - 3 > 2$.) So $(S - \{(a, a^h), (b, b^h)\}) \cup \{(a, b), (a^h, b^h)\}$ is a perfect matching of $AQ_k - F$, a contradiction. So the unique cross edge in $F_s$ must incident to either $u$ or $v$. The same applies to the unique complement edge in $F_c$. Since $AQ_k - F$ has no isolated vertices, we may assume the unique cross edge in $F_s$ is $(v, v^h)$ and the unique complement edge in $F_c$ is $(u, u^c)$. See Figure 7. It now follows from Proposition 2.2 that $v^h = u^c$ (which means that $v^c = u^h$). These edges of $F_s$ and $F_c$ form a trivial conditional matching preclusion set, with $v$ and $u$ adjacent only to vertex $v^c$ in $AQ_k - F$.

Fig. 7. $AQ^1_{k-1} - F_1$ has two isolated vertices.
We may now assume $AQ_{k-1}^1 - F_1$ has exactly one isolated vertex. Let this isolated vertex be $a$. We consider several subcases depending on the size of $|F_s| + |F_c|$.

**Subcase 1:** $|F_s| + |F_c| \geq 4$. Then $|F_1| + |F_0| \leq 4k - 9$. Since $|F_1| \geq 2k - 3$, $|F_0| \leq 2k - 6$. Since $AQ_n - F$ has no isolated vertices, at least one of $(a, a^h)$ and $(a, a^c)$ is not on $F$. Without loss of generality, assume $(a, a^h) \notin F$. We note that $|F_s| \leq 4k - 5 - |F_1| - |F_0| - |F_c| \leq 4k - 5 - (2k - 3) - 0 - 1 = 2k - 3$. Now, $a$ has $2k - 3$ neighbors. Suppose one of them is incident to a cross edge that is not in $F_s$. Call this neighbor $b$.

Let $F'_1 = F_1 - \{(a, b)\}$. Then $|F'_1| \leq 4k - 10$ and $AQ_{k-1}^1 - F''_1$ has no isolated vertices. So $AQ_{k-1}^1 - F''_1$ has a perfect matching $M''_1$ by the induction hypothesis and $M''_1$ contains $(a, b)$. As usual, we will extend $(M''_1 - \{(a, b)\}) \cup \{(a, a^h), (b, b^h)\}$ to a perfect matching in $AQ_k - F$. Let $F''_0$ be the set of edges in $AQ_{k-1}^0$ that are incident to $a^h$, and let $F''_0 = (F_0 \cup F'_0) - \{(a^b, b^h)\}$. Then $|F''_0| \leq (2k-6) + (2k-3) - 1 = 4k - 10$. By the structure of $F''_0$, it is easy to see that $AQ_{k-1}^0 - F''_0$ has no isolated vertices. So $AQ_{k-1}^0 - F''_0$ has a perfect matching $M''_0$ by the induction hypothesis and $M''_0$ contains $(a^b, b^h)$. Now, $(M''_1 - \{(a, b)\}) \cup \{(a, a^h), (b, b^h)\} \cup (M''_0 - \{(a^b, b^h)\})$ is a perfect matching of $AQ_k - F$ which gives a contradiction.

Such a $b$ exists unless $|F_s| \geq 2k - 3$ and $F_s$ contains the cross edge of every neighbor of $a$. But this means we have identified $F_1$, which is the set of edges incident to $a$ and we have identified $F_s$. Moreover $|F_c| = 1$ and $|F_0| = 0$. So consider any vertex $y$ in $AQ_{k-1}^1 - \{a, y\}$ that is neither $a$ nor a neighbor of $a$. Then $AQ_{k-1}^1 - \{a, y\}$ contains no edges from $F_1$. Thus apply Proposition 3.1 to obtain a perfect matching $M'_1$ of $AQ_{k-1}^1 - \{a, y\}$ and a perfect matching $M'_0$ of $AQ_{k-1}^0 - \{a^h, y^h\}$. Hence $M'_1 \cup M'_0 \cup \{(a, a^h), (y, y^h)\}$ is a perfect matching of $AQ_k - F$, a contradiction.

**Subcase 2:** $|F_s| + |F_c| = 3$. Then $|F_1| + |F_0| \leq 4k - 8$. Since $|F_1| \geq 2k - 3$, $|F_0| \leq 2k - 5$. Indeed, if we let $F'_1$ be the set obtained from $F_1$ by deleting all the edges incident to $a$, then $|F'_1| + |F_0| \leq 2k - 5$. Since $AQ_k - F$ has no isolated vertices, at least one of $(a, a^h)$ and $(a, a^c)$ is not in $F$. Without loss of generality, assume $(a, a^h) \notin F$. We note that $|F_s| \leq 4k - 5 - |F_1| - |F_0| - |F_c| \leq 4k - 5 - (2k - 3) - 0 - 1 = 2k - 3$. We consider several subcases.

**Subcase 2.1:** $|F'_1|, |F'_0| < 2k - 5$. Then $|F_1| \leq 2k - 3 + 2k - 6 = 4k - 9$ and $|F_0| \leq 2k - 6$. (So we can proceed as in Subcase 1.) Since $AQ_k - F$ has no isolated vertices, at least one of $(a, a^h)$ and $(a, a^c)$ is not on $F$. Without loss of generality, assume $(a, a^h) \notin F$. We note that $|F_s| \leq 2$. Now, $a$ has $2k - 3 \geq 7 > 2$ neighbors in $A^2_{k-1}$. Let $b$ be such a neighbor is incident to a cross edge that is not in $F_s$. Let $F''_1 = F_1 - \{(a, b)\}$. Then $|F''_1| \leq 4k - 10$ and $AQ_{k-1}^1 - F''_1$ has no isolated vertices. So $AQ_{k-1}^1 - F''_1$ has a perfect matching $M''_1$ by the induction hypothesis and $M''_1$ contains $(a, b)$. As usual, we will extend $(M''_1 - \{(a, b)\}) \cup \{(a, a^h), (b, b^h)\}$ to a perfect matching in $AQ_k - F$. Let $F''_0$ be the set of edges in $AQ_{k-1}^0$ that are incident to $a^h$, and let $F''_0 = (F_0 \cup F'_0) - \{(a^b, b^h)\}$. Then $|F''_0| \leq (2k-6) + (2k-3) - 1 = 4k - 10$. By the structure of $F''_0$, it is easy to see that $AQ_{k-1}^0 - F''_0$ has no isolated vertices. So $AQ_{k-1}^0 - F''_0$ has a perfect matching $M''_0$ by the induction hypothesis and $M''_0$
contains \((a^b, b^h)\). Now, \((M''_1^1 - \{(a, b)\}) \cup \{(a, a^b), (b, b^h)\} \cup (M''_1^0 - \{(a^h, b^h)\})\) is a perfect matching of \(AQ_k - F\) which gives a contradiction.

**Subcase 2.2**: \(|F'_1| = 2k - 5\). Then \(|F_0| = 0\). Since \(a\) has \(2k - 3 \geq 7\) neighbors and \(|F_s| \leq 2\), we can find two neighbors of \(a\), say \(b\) and \(c\) such that \((b, b^h), (c, a^h) \notin F_s\). Let \(F''_1 = F_1 - \{(a, b), (a, c)\}\). Then \(|F''_1| = (2k - 3) + (2k - 5) - 2 = 4k - 10\). Clearly \(AQ_{k-1}^1 - F''_1\) has no isolated vertices. So \(AQ_{k-1}^1 - F''_1\) has a perfect matching \(M''_1\) by the induction hypothesis and \(M''_1\) contains either \((a, b)\) or \((a, c)\). Without loss of generality, it contains \((a, b)\). By Proposition 2.1, \(AQ_{k-1}^0 - \{a^h, b^h\}\) has a perfect matching \(M''_0\). Then \((M''_0 - \{(a, b)\}) \cup \{(a, a^h), (b, b^h)\} \cup M''_1\) is a perfect matching of \(AQ_k - F\), a contradiction.

**Subcase 2.3**: \(|F_0| = 2k - 5\). Then \(|F_1| = 2k - 3\), that is, \(F_1\) consists of all the edges in \(AQ_{k-1}^1\) that are incident to \(a\). Since \(|F_s| \leq 2\), we can choose some neighbor of \(a\), say \(b\), such that \((b, b^h) \notin F_s\). Indeed, we have \(2k - 3 - 2 \geq 5\) choices for \(b\). Then \(AQ_{k-1}^1 - \{a, b\}\) contains no elements of \(F_1\). So by Proposition 3.1, \(AQ_{k-1}^1 - \{a, b\}\) has a perfect matching \(M_1\). Let \(F''_0 = (F_0 \cup F''_1) - \{(a^h, b^h)\}\). Then \(|F''_0| \leq (2k - 5) + (2k - 3) - 1 = 4k - 9\). We note that \(AQ_{k-1}^1 - F''_0\) does not have isolated vertices, as a vertex in \(AQ_{k-1}^1\) can be incident to at most 2 \(k - 4\) elements of \(F''_0\), but it has degree \(2k - 3\) in \(AQ_{k-1}^1\). So by the induction hypothesis, either \(AQ_{k-1}^1 - F''_0\) has a perfect matching or \(F''_0\) is a trivial conditional matching preclusion set in \(AQ_{k-1}^1\). If \(AQ_{k-1}^1 - F''_0\) has a perfect matching, then it must contain \((a^b, b^h)\), and hence we can use the usual trick to obtain a perfect matching of \(AQ_k - F\) which leads to a contradiction. Thus, we may assume that \(F''_0\) is a trivial conditional matching preclusion set in \(AQ_{k-1}^1\). So there must be some vertex \(d\) adjacent to \(a^h\) and \(b^h\) that now has no neighbors except for \(b^h\) in \(AQ_{k-1}^1 - F''_0\). See Figure 8. So \(F_0\) is completely determined, it contains the 2 \(k - 5\) edges, other than \((a^h, d)\) and \((b^h, d)\), that are incident to \(d\). Since we have at least 5 choices for \(b\), we will choose again, say \(c\), such that \(c^h \neq d\). Now repeat the argument, that is, let \(F''_0 = (F_0 \cup F''_1) - \{(a^b, d)\}\). It is now easy to see that \(F''_0\) is not a trivial conditional matching preclusion set in \(AQ_{k-1}^1\), regardless of whether \(c^h\) is adjacent to \(d\), as \((d, b^h) \notin F''_0\). So \(AQ_{k-1}^1 - F''_0\) has a perfect matching which will lead to a contradiction using the usual argument.

**Subcase 3**: \(|F_s| + |F_c| = 2\). So \(|F_s| = |F_c| = 1\) and \(|F_1| + |F_0| \leq 4k - 7\). Since \(|F_1| \geq 2k - 3\), \(|F_0| \leq 2k - 4\). Indeed, if we let \(F'_1\) be the set obtained from \(F_1\) by deleting all the edges incident to \(a\), then \(|F'_1| + |F_0| \leq 2k - 4\). We note that it follows from Proposition 2.2 that the unique cross edge in \(F_s\) and the unique complement edge in \(F_c\) are incident to a vertex \(y\). Since \(AQ_n - F\) has no isolated vertices, at least one of \((a, a^h)\) and \((a, a^h)\) is not on \(F\). Without loss of generality, assume \((a, a^h) \notin F\). We consider three possibilities.

**Subcase 3.1**: \(|F'_1|, |F'_0| < 2k - 4\). If \(|F'_1| \leq 2k - 6\), then it is essentially the same argument as in Subcase 2.1. If \(|F'_1| = 2k - 5\), then \(|F_1| = 4k - 8\) and \(|F_0| = 1\), so we will adjust the argument in Subcase 2.2 slightly. Since \(AQ_n - F\) has no isolated
vertices, at least one of \((a, a^h)\) and \((a, a^c)\) is not on \(F\). Without loss of generality, assume \((a, a^h) \notin F\). We recall that \(|F_s| = 1\). Now, \(a\) has \(2k - 3 \geq 7\) neighbors. Let \(b\) and \(c\) be such neighbors such that \((b, b^h), (c, c^h) \notin F_s\). Let \(F''_1 = F_1 - \{(a, b), (a, c)\}\). Then \(|F''_1| = 4k - 10\). Clearly \(AQ_{k-1}^1 - F''_1\) has no isolated vertices. So \(AQ_{k-1}^1 - F''_1\) has a perfect matching \(M''_1\) by the induction hypothesis and \(M''_1\) contains either \((a, b)\) or \((a, c)\). Without loss of generality, it contains \((a, b)\). If the unique element in \(F_0\) is \((a^h, b^h)\), the rest is the same as before. Otherwise, by Proposition 2.2, \(AQ_{k-1}^0\) has a perfect matching, \(M''_0\), containing the unique element in \(F_0\) and \((a^h, b^h)\). Then \((M''_0 - \{(a, b)\}) \cup \{(a, a^h), (b, b^h)\} \cup (M''_1 - \{(a^h, b^h)\})\) is a perfect matching of \(AQ_{k-1}^1 - F''_1\), a contradiction.

**Subcase 3.2:** \(|F'_1| = 2k - 4\). Then \(|F_1| = (2k - 3) + (2k - 4) = 4k - 7\). So \(|F_0| = 0\). Since \(a\) has \(2k - 3\) neighbors and \(|F_s| = 1\), there are at least \(2k - 3 - 1 = 2k - 4\geq 6\) neighbors of \(a\) whose unique cross edge that each is incident to is not in \(F_s\). Let \(b, c, d\) be three such neighbors. Let \(F''_1 = F_1 - \{(a, b), (a, c), (a, d)\}\). So \(|F''_1| = 4k - 10\). Clearly \(AQ_{k-1}^1 - F''_1\) has no isolated vertices. So \(AQ_{k-1}^1 - F''_1\) has a perfect matching \(M''_1\) by the induction hypothesis and \(M''_1\) contains exactly one of \((a, b), (a, c), (a, d)\). Without loss of generality, it contains \((a, b)\). By Proposition 2.1, \(AQ_{k-1}^0 - \{(a^h, b^h)\}\) has a perfect matching \(M''_0\). Then \((M''_0 - \{(a, b)\}) \cup \{(a, a^h), (b, b^h)\} \cup M''_1\) is a perfect matching of \(AQ_{k-1}^1 - F''_1\), a contradiction.

**Subcase 3.3:** \(|F_0| = 2k - 4\). Thus \(|F_1| = 2k - 3\), that is, \(F_1\) is the set of edges incident to \(a\) in \(AQ_{k-1}^1\). Since \(AQ_{k-1}^1 - F''_1\) has no isolated vertices, we may assume \((a, a^h) \notin F_s\). Let \((b, b^h)\) be the unique element of \(F_s\) where \(b\) is in \(AQ_{k-1}^1\). If there exists some neighbor \(v\) of \(b\) in \(AQ_{k-1}^1\) such that \((b, v), (b^h, v^h) \notin F\), then \((S - \{(b, b^h), (v, v^h)\}) \cup \{(b, v), (b^h, v^h)\}\) is a perfect matching of \(AQ_{k-1}^1 - F''_1\), a contradiction. Therefore, for each neighbor \(v\) of \(b\) in \(AQ_{k-1}^1\), either \((b, v) \in F\) or \((b^h, v^h) \in F\). Since \(b\) has \(2k - 3\) neighbors in \(AQ_{k-1}^1\) and \(|F_0| = 2k - 4\), the condition is satisfied unless \(b\) is adjacent to \(a\) in \(AQ_{k-1}^1\) and every edge incident to \(b^h\) in \(AQ_{k-1}^0\), except for
(b^h, a^h), is in \( F_0 \). See Figure 9. But we have now completely identified \( F \) except for the unique complement edge, \((z, z^c)\), in \( F_c \) where \( z \) is in \( AQ_{k-1}^1 \). We note that we do know \( z = b \) or \( z = b^h \) from Proposition 2.2.

We first consider the possibility that \( a^c = b^h \). This implies \( b^c = a^h \). If \( z = b^h \), then \((a, a^c) = (a, b^h) \in F \) and we have completely identified \( F \), which is a trivial conditional matching preclusion of \( AQ_k \). See Figure 10. If \( z = b \), then \((b, b^c) = (b, b^h) \in F \) and we have completely identified \( F \). See Figure 11. Let \( d \) be a neighbor of \( a \) in \( AQ_{k-1}^1 \) where \( d \neq b \). So \((a, d) \in F_1 \) and \((a^c, d^c) \in F_0 \). Moreover \( AQ_{k-1}^1 - \{a, d\} \) and \( AQ_{k-1}^0 - \{a^c, d^c\} \) have no elements from \( F \). So by Proposition 3.1, there is a perfect matching \( M''_1 \) in \( AQ_{k-1}^1 - \{a, d\} \) and a perfect matching \( M''_0 \) in \( AQ_{k-1}^0 - \{a^c, d^c\} \). Now, \( M''_1 \cup M''_0 \cup \{(a, a^c), (d, d^c)\} \) is a perfect matching of \( AQ_k - F \), a contradiction.

We may now assume that \( a^c \neq b^h \). So \((a, a^c) \notin F_c \). We find \( d \), a neighbor of \( a \) in \( AQ_{k-1}^1 \), such that \( d^c \neq a^h \) and \( d^c \neq b^h \). (Clearly this \( d \) exists.) See Figure 12. Now \((a^c, d^c)\) is not incident to \( b^h \). So \((a^h, b^h) \) and \((a^c, d^c)\) are independent. By Proposition 3.1, there is a perfect matching \( M''_1 \) in \( AQ_{k-1}^1 - \{a, d\} \). By Proposition 3.2, there is a perfect matching \( M''_0 \) in \( AQ_{k-1}^0 \) that contains \((a^h, b^h) \) and \((a^c, d^c)\). Clearly \( M''_0 \) does not contain any elements from \( F_0 \). Now, \( M''_1 \cup (M''_0 - \{(a^c, d^c)\}) \cup \{(a, a^c), (d, d^c)\} \) is a perfect matching of \( AQ_k - F \), a contradiction. This completes the proof. \( \square \)
Fig. 10. A trivial conditional matching preclusion set.

Fig. 11. $z = b$.

Fig. 12. $a^c \neq b^h$. 
4. Conclusion

The augmented cubes were introduced as attractive variants of the hypercubes, and indeed, they are superior in many ways. In this paper, we determined their matching preclusion number and their conditional matching preclusion number. Moreover, we classified the optimal matching preclusion sets and the optimal conditional matching preclusion sets. Since a Hamiltonian cycle gives two edge-disjoint perfect matchings, one approach is to leverage on existing results on fault Hamiltonicity proved by Hsu et al.\(^{10}\) However, the proofs are rather involved and we choose to not use them so that we can present a proof that is self-contained. The computer program used in the proof of Lemma 3.2 generates all cases in Step 1 and use a standard primal-dual algorithm to find a perfect matching. The program is written in C. Step 1 was completed in about two hours on a computer with a 2.16 GHz Intel Core 2 Duo processor and 2 GB of RAM.

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Appendix A. Proof of Lemma 3.2

The proof is similar to the proof given in Theorem 3.1 when the induction step is given, but obviously there are differences, as we are not using induction since the claim is not true for \(AQ_3\). So the first step is the following lemma. Throughout this appendix, we define \(AQ_i\)'s, \(F_1, F_0, F_s, F_c\) as usual.

**Lemma Appendix A.1.** Let \(F\) be a set of edges of size 11 such that \(AQ_4 - F\) has no isolated vertices. Let \(F_i\) be either \(F_1\) or \(F_0\). If \(|F_1| \geq 6\) and \(F_i\) contains the conditional matching preclusion set for \(AQ_3\) given in Figure 3, then \(AQ_4 - F\) has a perfect matching; if \(|F_1| \geq 7\) and \(F_i\) contains the conditional matching preclusion set for \(AQ_3\) given in Figure 4, Figure 5 or Figure 6, then \(AQ_4 - F\) has a perfect matching.

**Proof.** We established this via a computer program. \(\Box\)

Now, let \(F\) be a set of edges of size 11 such that \(AQ_4 - F\) has no isolated vertices. We may assume \(|F_0|, |F_1| \geq 1\). We consider whether \(AQ_4^0 - F_0\) and \(AQ_4^1 - F_1\) have isolated vertices. These two cases correspond to Lemma Appendix A.2 and Lemma Appendix A.3. The proof of Lemma Appendix A.2 is more tedious. The proof of Lemma Appendix A.3 is much simpler by using Lemma Appendix A.1. We remark that although this proof does not use the full strength of Lemma Appendix A.1, Lemma Appendix A.1 is presented in its present form because it is interesting in its own right.

**Lemma Appendix A.2.** Let \(F\) be a set of edges of size 11 such that \(AQ_4 - F\) has no isolated vertices. Suppose either \(AQ_4^1 - F_1\) or \(AQ_4^0 - F_0\) contains isolated
vertices. Then either $F$ is a trivial conditional matching preclusion set or $AQ_4 - F$ has a perfect matching.

**Proof.** Suppose the statement is not true. Then as in the proof in Case 2 of the proof of Theorem 3.1, such an isolated vertex is unique. Assume without loss of generality that $AQ_3^0 - F_0$ has vertex 0000 isolated. Let $T$ be the 5 edges incident to 0000 in $AQ_3^0$. Then $T \subseteq F_1$. So $|F - T| = 6$. Now, we know $|F_c|, |F_s| \geq 1$. We consider two cases.

**Case 1:** $|F_c|, |F_s| \geq 2$.

*Subcase 1.1:* $|F_c| + |F_s| = 6$. Then $|F_0 - T| = 0$ and $|F_1| = 0$. Since 0000 is not isolated in $AQ_4 - F$, one can match 0000 to a neighbor in $AQ_3^0$ using an edge $e$ not in $F$. Now pick any other non-faulty cross or complement edge that is independent of $e$. Apply Proposition 3.1 to obtain a perfect matching in $AQ_4 - F$.

*Subcase 1.2:* $|F_c| + |F_s| = 5$. Then $|F_0 - T| + |F_1| = 1$. Note that $|F_c|, |F_s| \leq 4$. Assume without loss of generality that the cross edge of 0000 is not in $F$. Find a neighbor of 0000 in $AQ_3^0$ whose cross edge is not in $F$, say $a$. (It exists since $|F_s| \leq 4$.) We claim that $AQ_4 - F$ has a perfect matching. We use the edges $(0000, 1000)$ and $(a, a' b')$. We claim that there is a perfect matching $M_0$ in $AQ_3^0 - (F_0 \cup \{0000, a\})$. Recall that $|F_0 - T| \leq 1$. If $|F_0 - T| = 0$, then apply Proposition 3.1 using $(0000, a)$. Suppose $|F_0 - T| = 1$ and $f$ is the unique edge in $F_1 - T_1$. Pick an edge $g$ that shares a vertex with $f$ but is independent of $(0000, a)$. Now apply Proposition 3.2 using $(0000, a)$ and $g$. Similarly, there is a perfect matching $M_1$ in $AQ_3^1 - (F_1 \cup \{1000, a' b\})$.

Now $(\{0000, 1000\}, (a, a' b')) \cup M_0 \cup M_1$ is a perfect matching in $AQ_4 - F$.

*Subcase 1.3:* $|F_c| + |F_s| = 4$. Then $|F_c| = |F_s| = 2$. Therefore, $|F_0 - T| + |F_1| = 2$. Assume without loss of generality that the cross edge of 0000 is not in $F$. Then we claim that the cross edge of 0010 must be in $F$. If not, then Figure 13 gives three edge-disjoint perfect matchings of $AQ_4 - (\{0000, 0010, 1000, 1010\} \cup F_c \cup F_s)$. Since $|F_0 - T| + |F_1| = 2$ and $(0010, 1010)$ is not in $F$, $AQ_4 - F$ has a perfect matching. The same argument applies to the cross edge of 0001 using Figure 14 and the cross edge of 0100 using Figure 15, leading to a contradiction.

**Case 2:** $|F_s| = 1$ or $|F_c| = 1$. Without loss of generality, assume $|F_s| = 1$. Hence $|F_c| = 1, 2, 3, 4$ or 5.

*Subcase 2.1:* $|F_c| = 1$. So $|F_s| = |F_c| = 1$. Now, since $F$ must contain the 5 edges incident to 0000 (again, call these edges $T$), $|F_0 - T| + |F_1| = 4$. Either exactly one of the cross edge and the complement edge of 0000 is in $F$ or neither is.

*Subcase 2.1.1:* Exactly one of the cross edge and the complement edge of 0000 is in $F$. Then assume without loss of generality that the cross edge of 0000 is in $F$. If the unique element of $F_c$ is not (0111, 1000), then $AQ_4 - F$ has a perfect matching consisting of $(0000, 1111), (0111, 1000)$, and the appropriate cross edges of $AQ_4$. Hence the edge in $F_c$ must be $(0111, 1000)$.

We now place constraints on the 4 edges in $(F_0 - T) \cup F_1$. If there exists some neighbor $a \in AQ_3^0$ of 0111 such that neither $(a, 0111)$ nor $(a', 1000)$ is in $F$, then a perfect matching of $AQ_4$ consists of $(a, 0111), (a', 1000)$, and the appropriate
Fig. 13. $(0010, 1010) \in F$ or we get three perfect matchings with distinct edges.

Fig. 14. $(0001, 1001) \in F$ or we get three perfect matchings again.

complement edges of $AQ_4$. We notice that there are four choices of $a$. (Although 0111 has 5 neighbors in $AQ^0_3$, one of them is 0000.) Hence, each of the 4 edges in $(F_0 - T) \cup F_1$ must be incident to either 0111 or 1000. If all of these four edges are incident to 0111, it violates our assumption that there is only one vertex isolated in $AQ^1_3 - F_1$ and $AQ^0_3 - F_0$. If all of these four edges are incident to 1000, $F$ becomes a trivial conditional matching preclusion. Therefore we assume that 0111 is not isolated in $AQ^0_3 - F_0$ and 1000 is not isolated in $AQ^1_3 - F_1$. Now, find some arbitrary neighbor of 0111 in $AQ^0_3 - F_0$, say $b$, and find some neighbor of 1000 in $AQ^1_3 - F_1$, say $c$. We construct a required perfect matching of $AQ_4 - F$ by including $(0111, b), (1000, c), (0010, 1101), (0000, 1111)$. We then complete the perfect matching applying Proposition 3.2, using $(0111, b)$ and $(0010, 0000)$ for $AQ^0_3$ and $(1000, c), (1101, 1111)$ for $AQ^1_3$. (We can apply Proposition 3.2 since each of the 4 edges in $(F_0 - T) \cup F_1$ must be incident to either 0111 or 1000.)
Subcase 2.1.2: Neither the cross nor the complement edge of 0000 is in $F$. Call the edge in $F_s$ $(a, a^h)$ where $a \in AQ^0_3$. Notice that if there exists some neighbor $b \in AQ^0_3$ of $a$ such that neither $(a, b)$ nor $(a^h, b^h)$ is in $F$, then a perfect matching of $AQ_4$ consists of $(a, b), (a^h, b^h)$ and the appropriate cross edges. If vertex $a$ is not incident to 0000, then $a$ has five choices for $b$ while only four edges incident to $a$ can be in $F$. Therefore, vertex $a$ is a neighbor of 0000 in $AQ^0_3$ and each of the four edges in $(F_0 - T) \cup F_1$ must be incident to either $a$ or $a^h$.

Subcase 2.1.2.1: $a = 0111$. If the complement edge of $a$ is not in $F$, then we obtain a perfect matching of $AQ_4 - F$ consisting of $(a, 1000), (a^h, 0000)$ and the appropriate cross edges. If the complement edge of $a$ is in $F$, then find some neighbor of $a$ in $AQ^0_3 - F_0$, say $u$. We construct a perfect matching of $AQ_4 - F$ by including edges $(a^h, 0000), (a, u)$ and $(0001, 1001)$. We can complete the perfect matching by applying Proposition 3.2 to $AQ^0_3$, using $(0111, u)$ and $(0000, 0001)$, and by applying Proposition 3.1 to $AQ^1_3$, using 1111 and 1001. (Again, Proposition 3.1 and Proposition 3.2 are applicable since each of the 4 edges in $(F_0 - T) \cup F_1$ must be incident to either $a = 0111$ or $a^h = 1111$.

Subcase 2.1.2.2: $a \neq 0111$. The edges of $F_0 - T$ cannot all be incident to $a$ because this would isolate $a$ in $AQ^0_3$ and violate our assumption. If some of these four edges are not incident to $a^h$, then we find some neighbor of 0000 in $AQ^0_3$, say $c$, that is neither $a$ nor adjacent to $a$. Then, find neighbor $d$ of $a$ in $AQ^0_3 - F_0$ and find neighbor $e$ of $a^h$ in $AQ^1_3 - F_1$. We can begin a perfect matching of the graph by taking edges $(0000, 1000), (c, c^h), (a, d)$ and $(a^h, e)$. Applying Proposition 3.2 to $AQ^0_3$, using $(0000, c)$ and $(a, d)$, and to $AQ^1_3$, using $(1000, c^h)$ and $(a^h, c)$, completes the perfect matching. (Again, Proposition 3.2 is applicable since each of the 4 edges in $(F_0 - T) \cup F_1$ must be incident to either $a$ or $a^h$.)

Subcase 2.2: $|F_c| \geq 2$. Recall that $|F_s| = 1$. We claim that the cross edge of 0000 must be in $F$. If it is not, then call the cross edge in $F_c$ $(a, a^h)$ where $a \in AQ^0_3$. For each neighbor $b \neq 0000$ of $a$, either $(a, b)$ or $(a^h, b^h)$ is in $F$ or $(a, b), (a^h, b^h)$, and
the appropriate cross edges form a perfect matching in $AQ_4 - F$. However, there are at least four neighbors $b$, but $|F_0 - T| + |F_1| \leq 3$. Therefore the cross edge of 0000 is in $F$. Since no vertices are isolated in $AQ_4 - F$, this means the complement edge of 0000 is not in $F$. Henceforth, we may assume the cross edge at 0000 is in $F$, that is, $F_s = \{(0000, 1000)\}$ but the complement edge at 0000 is not in $F$, that is, $(0000, 1111) \notin F_c$.

If $|F_c| = 2$, then one of these edges must be $(1000, 0111)$ or else $(0000, 1111), (1000, 0111)$, and the appropriate cross edges give a perfect matching of $AQ_4 - F$. Recall that here we have $|F_0 - T| + |F_1| = 3$ and $F_s = \{(0000, 1000)\}$. If edge $(0001, 1110)$ is not in $F$ then we get four edge-disjoint perfect matchings of $AQ_4 - \{(0000, 1111, 0001, 1110) \cup F_c \cup F_s\}$ as given in Figure 16, so $(0001, 1110)$ must be in $F$. Similarly, $(0010, 1101)$ must be in $F$. (See Figure 17.) But this contradicts the fact that $|F_c| = 2$.

If $|F_c| = 3$, $F_c$ must contain $(0111, 1000), (0001, 1110)$ and $(0010, 1101)$ by the above argument. Additionally, we now have $|F_0 - T| + |F_1| = 2$. We claim that $(0100, 1011)$ must be in $F$, or else we get three edge-disjoint perfect matchings of $AQ_4 - \{(0000, 1111, 0100, 1011) \cup F_c \cup F_s\}$. (See Figure 18.) However, $|F_c| = 3$, which gives a contradiction.
Fig. 18. Three perfect matchings of edges not in $F$ and distinct $AQ_3$ edges if $(0100, 1011)$ is not in $F$.

Fig. 19. Three perfect matchings of edges not in $F$ and distinct $AQ_3$ edges if $(0011, 1100)$ is not in $F$.

If $|F_c| = 4$, then $F_c$ must contain $(0111, 1000)$, $(0001, 1110)$, $(0010, 1101)$ and $(0100, 1011)$ by the above argument. But we now have $|F_0| + |F_1| = 1$. We claim that $(0011, 1100)$ must be in $F$, or we get two edge-disjoint perfect matchings of $AQ_4 - (\{0000, 1111, 0011, 1100\} \cup F_c \cup F_s)$. (See Figure 19.) Again, this contradicts our assumption that $|F_c| = 4$.

Hence $|F_c| = 5$ and $F_0 = F_1 = \emptyset$. We match 0000 to 1111, use any other non-faulty cross or complement edge, and apply Proposition 3.1 in $AQ_3^0$ and $AQ_3^1$ to find a perfect matching of $AQ_4 - F$.

**Lemma Appendix A.3.** Let $F$ be a set of edges of size 11 such that $AQ_4 - F$ has no isolated vertices. Suppose both $AQ_3^1 - F_1$ and $AQ_3^0 - F_0$ have no isolated vertices. Then either $F$ is a trivial conditional matching preclusion set or $AQ_4 - F$ has a perfect matching.
Proof. Without loss of generality, assume $AQ_3^0 - F_0$ has no perfect matching. It follows from Lemma 3.1 and Lemma Appendix A.1 that $|F_0| \geq 7$. Since $|F_e|, |F_c| \geq 1$, $|F_0| = 7, 8$ or $9$. Before we continue with the proof, we remark that the same program that verified Lemma Appendix A.1 is used to prove the following: Let $F$ be a set of edges of size $11$ such that $AQ_4 - F$ has no isolated vertices and $|F_0| \geq 7$. If $F_0$ contains a trivial conditional matching preclusion set in $AQ_3^0$, then $F$ is either a trivial conditional matching preclusion set in $AQ_4$ or $AQ_4 - F$ has a perfect matching. This can be used to simplify the proof but we will nevertheless give a theoretical justification.

Case 1: $|F_0| = 7$. Then it follows from Lemma 3.1 and Lemma Appendix A.1 that $F_0$ must be a trivial conditional matching preclusion set, that is, there is a 2-path $a - c - b$ such that $F_0$ consists of the edges incident to either $a$ or $b$, except the edges $(a, c)$ and $(b, c)$. Note that $(a, b)$ is in $AQ_3^0$. Indeed, we can now apply the above fact which we checked with a computer program to finish this case. Nevertheless, we proceed with a theoretical argument. If one of $|F_e|, |F_c| = 1$, assume without loss of generality that $|F_s| = 1$ and that the cross edge of $a$ is not in $F$. Suppose $|F_s| = |F_c| = 2$. If all the cross and complement edges of $a$ and $c$ are in $F$, then $F$ becomes a trivial conditional matching preclusion set. Therefore without loss of generality assume the cross edge of $a$ is not in $F$. There is a neighbor $d \neq c$ of $a$ with a cross edge that is not in $F$. Match $a$ with $a^h$ and $d$ with $d^h$. Define $T$ to be the set of edges of $AQ_3^1$ incident to $a^h$ not including $(a^h, d^h)$. Let $F'_1 = (F_1 \cup T) - \{(a^h, d^h)\}$. Notice that $AQ_3^1 - \{(a^h, d^h)\} \cup F_1$ has a perfect matching if and only if $AQ_3^1 - F'_1$ does. Since $|F'_1| \leq 6$, $AQ_3^1 - F'_1$ has a perfect matching unless $F'_1$ is the nontrivial conditional matching preclusion set shown in Figure 3. By examining this conditional matching preclusion set, we notice that $(a^h, d^h)$ must have been one of $(1000, 0101), (1010, 1001), (1100, 1111), \text{or} (1110, 1101)$. We notice that we can simply pick a different $d$ to use, since $a$ has at least two adjacent vertices $d$ such that $(a, d)$ is in $F$ and $(d, d^h)$ is not. This will give us a new $(a^h, d^h)$ that cannot be one of $(1000, 1011), (1010, 1001), (1100, 1111), \text{or} (1110, 1101)$. Therefore we will find a perfect matching of $AQ_3^1 - F_1$. Now $(AQ_3^0 - (a, d)) - F_0$ has a perfect matching. (Use Proposition 3.2 with $(a, d)$ and $(b, c)$.) Thus $AQ_4$ has a perfect matching.

Case 2: $|F_0| = 8$. Assume without loss of generality that $|F_s| = 1$. There exists some edge $(u, v)$ in $F_0$ with $(u, u^h), (v, v^h)$ both not in $F$. Consider set $F'_0 = F_0 - \{(u, v)\}$. We consider two subcases.

Subcase 2.1: $AQ_3^0 - F'_0$ has a perfect matching $M_0$. Then $(u, v) \in M_0$. Now $|F_1| \leq 1$. If $|F_1| = 1$ and the unique edge in $F_1$, say $(p, q)$, is independent to $(u^h, v^h)$, then we pick an edge $(p, r)$ in $AQ_3^1 - F_1$ where $r \notin \{u^h, v^h, q\}$, and use Proposition 3.2 to get a perfect matching $M_1$ in $AQ_3^1 - ((u^h, v^h) \cup \{(p, q)\})$; otherwise, we use Proposition 3.1 to get a perfect matching $M_1$ in $AQ_3^1 - ((u^h, v^h) \cup F_1)$ using $u^h$ and $v^h$. Now $(M_0 - \{(u, v)\}) \cup M_1 \cup \{(u, u^h), (v, v^h)\}$ is a perfect matching in $AQ_4 - F$. 
Subcase 2.2: If $AQ_3^0 - F_0$ does not have a perfect matching, then it follows from Lemma 3.1 and Lemma Appendix A.1 that $F_0'$ must be a trivial conditional matching preclusion set; that is, there is a 2-path $a - c - b$ such that $F_0$ consists of the edges incident to either $a$ or $b$, except the edges $(a, c)$ and $(b, c)$. Note that $(a, b)$ is in $AQ_3^0$. Again, we can now apply the above fact which we checked with a computer program to finish this subcase. However, we will give a theoretical argument. At least one of $a, b$ has its cross edge not in $F$. Without loss of generality, say it is $a$. Then, four edges of $F_0$ are incident to $a$. (One of them is $(a, b)$.) At least three neighbors of $a$, say one is $d$, satisfy the properties that $(a, d)$ is in $F$ and $(d, d^b)$ is not in $F$. It is easy to see that we can pick one such $(a, d)$ such that $F_0 - \{(a, d)\}$ is not a trivial conditional matching preclusion set in $AQ_3^0$. If $AQ_3^0 - (F_0 - \{(a, d)\})$ has no perfect matchings, then $F_0 - \{(a, d)\}$ contains the set of shaded edges in Figure 3, or $F$ is the set of shaded edges in Figure 4, Figure 5 or Figure 6, up to automorphism. But by Lemma Appendix A.1, $AQ_4 - F$ has a perfect matching. So we may assume $AQ_3^0 - (F_0 - \{(a, d)\})$ has a perfect matching, which is covered by Subcase 2.1.

Case 3: $|F_0| = 9$. So $|F_s| = |F_c| = 1$ and $|F_1| = 0$. Consider the unique cross edge in $F$, $(a, a^h)$ where $a \in AQ_3^0$. Since $AQ_3^0 - F_0$ has no isolated vertices, pick $(a, c)$ in $AQ_3^0 - F_0$. Now, $(a, c)$ and $(a^h, c^b)$ together with appropriate cross edges give a perfect matching in $AQ_4 - F$.

Now Lemma 3.2 follows from Lemma Appendix A.2 and Lemma Appendix A.3.

References


