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Saturday, October 27, 2018

Queensborough Community College
Bayside, NY

Conference Theme

The Art of the Neuron

Keynote Addresses

will be presented by

Alison Dell
and
Gregory Dunn

Instructions for Authors

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51st Annual Fall MACUB Conference
Saturday, October 27, 2018, 8:30 A.M.-4 P.M.
Queensborough Community College, Bayside, NY
Conference theme:

Art of the Neuron

Gregory Dunn, Ph.D.

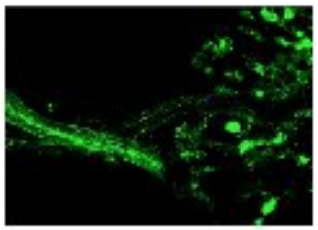
Doctorate from the University of Pennsylvania
Artist
http:// www.gregadunn.com/

Dr. Dunn has used his Ph.D. in Neuroscience and his art background to help him focus on making art about the brain and neurons. He is especially interested in microetching, a technique which he adds an additional animated dimension to his artwork by precisely controlling the reflective properties of gold surfaces.

Alison Dell, Ph.D.

Assistant Professor, Department of Biology, Health Promotion, and Health Care Management
St. Francis College, Brooklyn, NY

Dr. Dell's research interests include cell signaling in neural development, as well as the impact of common environmental toxins on these processes. She is the co-founder of Art in the Lab, an ongoing series bringing scientists and artists together in lab for workshops that combine drawing and laboratory exercises.



Homology Modeling of Glial Fibrillary Acidic Protein and Functional Prediction in Alexander Disease

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Abstract

Alexander disease (AxD) is an autosomal dominant neurodegenerative disease caused by a point mutation in the glial fibrillary acidic protein (GFAP) gene. GFAP is a Type III intermediate filament protein exclusively expressed in the astrocytes of the adult CNS. The most commonly known function of GFAP is to provide the mechanical strength needed to maintain cell shape. Due to the insolubility of GFAP *in vitro* and the lack of proper crystallizing methodology for intermediate filaments, it has long been challenging to study GFAP structures using standard x-ray diffraction techniques or nuclear magnetic resonance spectroscopic techniques. Here we show an alternative method that utilizes mainstream online protein databases for sequence and structural analysis of the GFAP. We first determined the amino acid sequence of the GFAP based on NCBI nucleotide sequence and identified its close homologues through BLAST. Then we proposed a coiled coil motif for the multimerization domain of GFAP based on its sequence similarity with that of vimentin whose crystal structure has been solved. As a first step toward understanding the structure-function relationship of GFAP, we comparatively examined the effect of hydrophobicity, size, and polarity of amino acid 239 on the coiled coil formation of GFAP. We predicted that R239 substitution in the 2A segment of GFAP, known as the hotspot mutation of AxD, has a significantly low probability of forming coiled-coil heptad repeat motif. Our predictions are consistent with insights gained from studies using different biochemical and biophysical approaches that altered oligomerization kinetics of R239 mutation contributes to more intermediate and aggregated forms of GFAP in AxD. Further, we built the first conceptual model of GFAP 3D structure upon the crystal structure of vimentin. Our study provides the first attempt of correlating GFAP function and stability through structure homology modeling. The conceptual model presented here and further computational analysis may contribute to the development of structure-based therapeutic interventions for AxD.

Keywords: Alexander disease, astrocyte, intermediate filament, glial fibrillary acidic protein, vimentin, segment assembly, coiled coil, structure alignment, protein modeling

Introduction

Alexander disease (AxD), caused by point mutations of glial fibrillary acidic protein (GFAP) in astrocytes, is a devastating disorder of the central nervous system associated with impaired physical and mental development, seizures, and megalencephaly among other serious conditions[1,2] . GFAP is an intermediate filament protein and thought to play a role in astrocyte-neuron interactions[3] as well as maintaining the strength of the astrocytes[4]. One proposed model is that mutant GFAP slows the formation of normal intermediate filaments, resulting in the accumulation of its oligomers[5-8]. Identifying the effects of GFAP mutations on its 3D conformation is therefore the key in understanding the pathogenesis of AxD. However, high-resolution X -ray studies of GFAP structure is currently unsuccessful due to lack of its crytallizing methodology. While crystallization for most intermediate filaments remains a challenge, the structure of human vimentin was successfully determined by crystallization of its subfragments and overlap alignment[9]. Vimentin is also a type III intermediate filament protein showing close sequence homology to GFAP[10] and a major cytoskeletal component in the newborn CNS. In this report, we show that GFAP and vimentin share common amino acid sequences in their 2A segments, where most AxD-causing mutations are discovered. Furthermore, various tools of bioinformatics allow us to construct a conceptual model of the antiparallel tetramer representing the second level of GFAP assembly.

Materials and Methods

Sequencing

The FAFTA format sequence of wild type human GFAP was obtained from the NCBI gene database. The translated amino acid sequence of GFAP was obtained from its reading frame through The Sequence Manipulation Suite (www.bioinformatics.org/sms/), as described previously in detail[8]. Other web-based tools of analysis included T-Coffee (tcoffee.org, Center for Genomic Regulation, Barcelona) for multiple sequence alignment.

BLAST Analysis

The structural framework of GFAP (UnitPort ID P14136; under Protein Feature View) and the sequence motif of its domain 2A were obtained from RCBS Protein Data Bank (www.rcsb.org)[11]. The amino acid sequence of domain 2A was compared against other type III intermediate filament proteins, namely, vimentin (P08670), desmin (P17661), and peripherin (P41219) by BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi, with sequence alignment option).

3-D Modeling

Cn3D (version 4.3), a visualizing software of molecular structure from NCBI, was utilized to obtain 3D images of vimentin and to identify its regions containing amino acid sequences homologous to GFAP (www.ncbi. nlm.nih.gov/ Structure/CN3D/cn3d.shtml)[12]. A 3D model of GFAP was generated by superimposing GFAP homologues onto the crystal structure of vimentin using UCSF Chimera package, a molecular graphics and analyses software (www.cgl.ucsf.edu/chimera) developed by the Resource for Biocomputing, Visualization, and Informatics at UCSF[13].

Results

BLAST Search for GFAP Homology

BLAST alignment analysis of amino acids carried out between GFAP and all other type III intermediate filaments. Results revealed relatively high degree of homology consisting of 76% for vimentin coil 2, 75% for desmin coil 2A, and 76% for peripherin coil 2 (Table 1). Of the three, only the 3D structure of vimentin was characterized by x-ray crystallography and therefore was pursued further.

Use of Vimentin as the Reference Structure of GFAP

Currently the 3D structure of GFAP is unavailable in NCBI as it has not been characterized by crystallographic methods. As

Table 1 Result of BLAST homology analysis by amino acid alignment for coil 2A of vimentin, desmin, or peripherin against WT GFAP coil 2A.

Coil 2A of:	Sequences Q: Query S: Subject	Score	Expect	Identities	Positives	Gaps
GFAP Vimentin	Q: 1 DLTAALKEIRTQYEAMA 17 DLTAAL ++R QYE A S: 1 DLTAALRDVRQQYESVA 17	36.7 bits (79)	4e-11	11/17 (65%)	13/17 (76%)	0/17 (0%)
GFAP Desmin	Q: 1 LTAALKEIRTQYEAMA 16 LTAAL +IR QYE A S: 2 LTAALRDIRAQYETIA 17	37.1 bits (80)	4e-11	11/16 (69%)	12/16 (75%)	0/16 (0%)
GFAP Peripherin	Q: 1 DLTAALKEIRTQYEAMA 17 +LTAAL +IR QYE A S: 1 ELTAALRDIRAQYESIA 17	38.4 bits (83)	1e-11	11/17 (65%)	13/17 (76%)	0/17 (0%)

mentioned above, however, several crystallographic 3D models of vimentin subfragments are available. Among these, a tetrameric structure of vimentin coil 2 was examined in detail for comparative analysis against GFAP (Fig. 1). The tetramer was visualized through "space fill" rendering in Cn3D that shows the distribution of its individual amino acids (Fig. 1A,B). The four subunits of the tetramer, each consisting of an alpha helix, represented in color, are intertwined with one another along the longitudinal axis, a pattern characteristics of coiled coil folding motif. The extent of structural homology between vimentin coil 2, now serving as a reference structure, and WT GFAP was assessed by highlighting their shared amino residues, which is visually extensive all shared residues highlighted in yellow (Fig 1C,D).

Coiled Coil Motif of Vimentin

Coiled coil folding motifs consist of two to five alpha helices that are intertwined with one another forming a supercoil[14]. Visualization of vimentin through "tube" rendering in Cn3D produced a more obvious representation of its alpha helices, with a left twist typical of coiled coils (Fig. 2a). Amino acids in the alpha helices of coiled coil are known to occur in series of seven residues known as a heptad (Fig. 2b)[15]. Each residue is assigned a position letter that is associated with properties necessary in generating the coiled coil motif.

Coiled Coil Prediction in WT and R239 mutant GFAP in AxD

The probability of forming coiled coil motif within WT GFAP (wildtype) was obtained by evaluating its ORF amino sequence through web-based program COILS. The result yielded a probability value of 0.689 at amino acid 239 with a sliding window size of 14 (or at a resolution of 2 heptads, green plot; (Fig. 3 left). However, when the R239C AxD sequence was evaluated, the resulting probability decreased to approximately 0.1 (Fig. 3 right). The affinity of residues [a] and [d] in heptads maintains the stability of coiled coil motif at its core region. For WT GFAP domain 2A, these amino acids were identified as leucine, isoleucine, tyrosine, and methionine, which are all examples of hydrophobic, large, and nonpolar amino acids except for tyrosine (Table 2). The prediction further supports that coiled coil formation is significantly disturbed by R239 mutation.

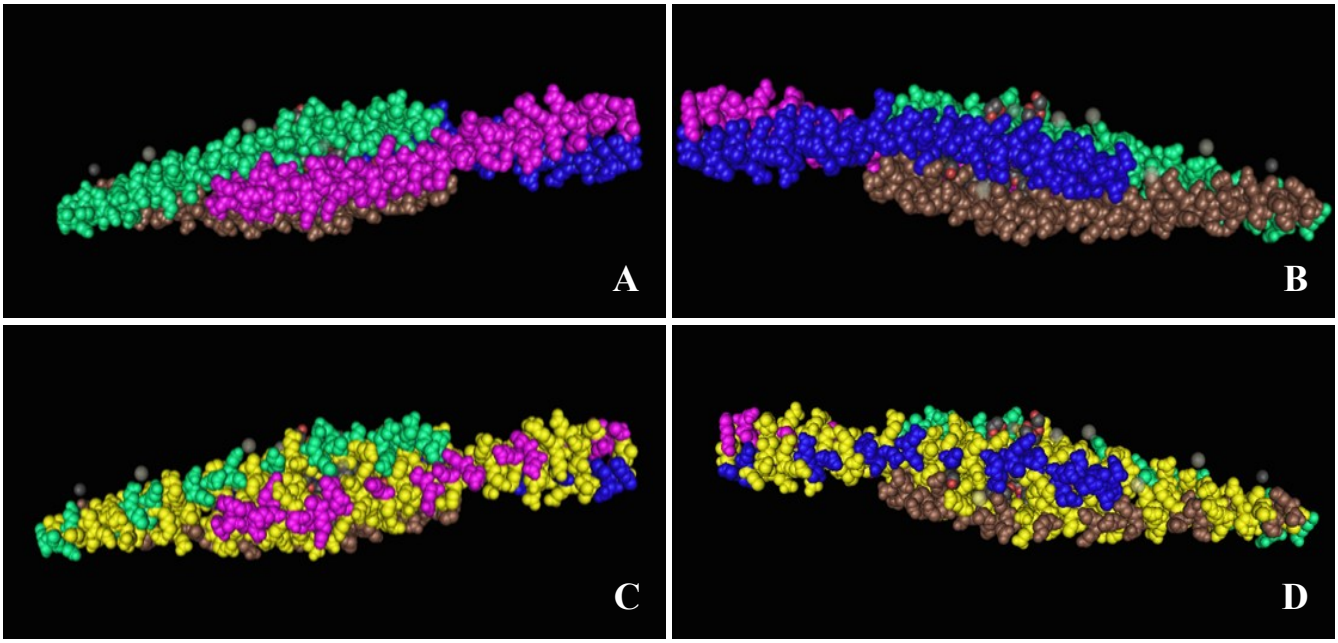

Fig. 1 Tetramer of vimentin coil 2 as shown by Cn3D software (panels A and B, lateral views). Each of the subunits in the tetramer consist of the amino acid sequence: LRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTEYRRQVQSLTCEVDALK. Amino acids in red are shared residues between vimentin and GFAP and are highlighted in yellow, respectively in panels C and D.

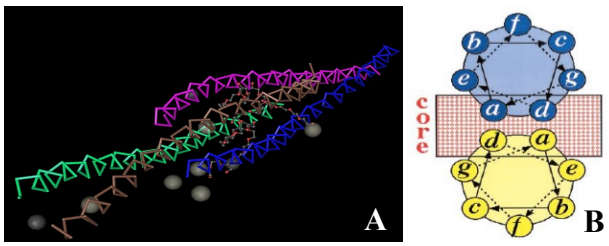

Fig. 2 3D structure of vimentin showing its tetramers arranged into coiled coil motif (A). Pattern of heptads, or repeating series seven amino acids, within each alpha helix of coiled coil motif. The helices interact through hydrophobic amino acids at positions [a] and [d]

Effect of Changes in Hydrophobicity and Size of Residue at Position 239 on the Formation of Coiled Coil in GFAP

In WT GFAP, R239 is located at position [e] of predicted coiled coil 2A motif. The supercoil is expected to arise from the interaction between the side-chain of residue [e] in domain 2A of one subunit and the side-chain of residue [g] in domain 2B of the partner subunit (not taking into account the other two subunits for simplification). As both [e] and [g] are polar residues in WT, it is expected that changes in the hydrophobicity and the size of these amino acids can affect their electrostatic interaction, and thus the formation of the coiled coil. Table 3 lists the hydrophobicity, size, and polarity of all other 19 amino acids and the result of substituting R239 with each one. In summary, substitutions that permit coiled coil formation include K, N, D, Q, and E, while the rest indicate inhibition. The permissive amino acids are all hydrophilic, large, and polar types as arginine.

3D Structure of Human GFAP

Currently there is no reported crystal structure of GFAP. In an effort to arrive at a more tangible conceptualization of GFAP structure, a 3D model of the protein was built upon known crystal structure of its

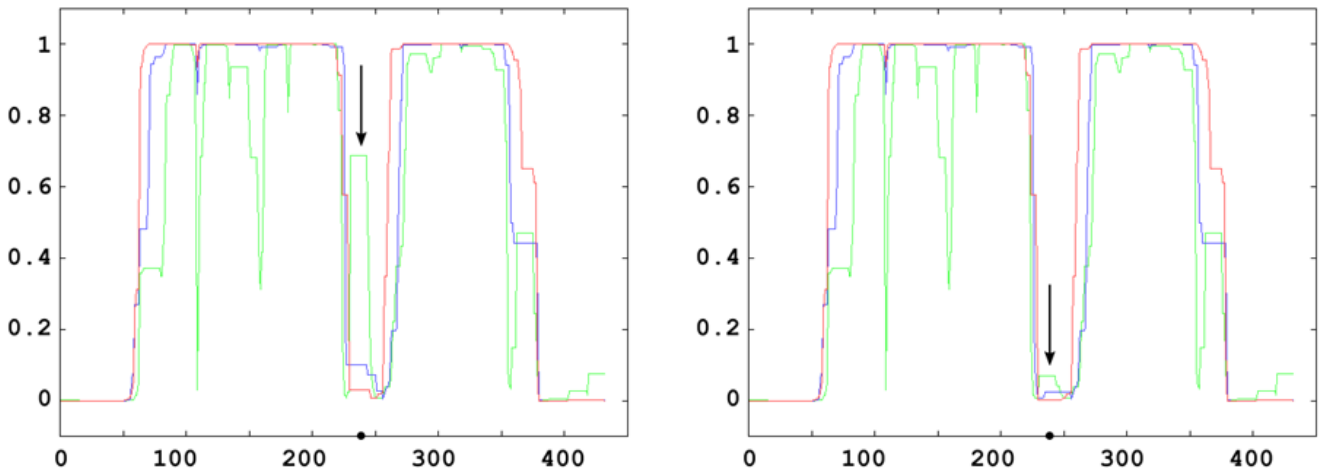

Fig. 3 Coiled coil probability of amino acid 239 (dot) for wild type (left) and mutant GFAP (right). Sliding window sizes are 28 residues (or 4 heptads in red), 21 residues (or 3 heptads in blue), and 14 residues (or 2 heptads in green). Differences are noticeable for window=14 (arrow)

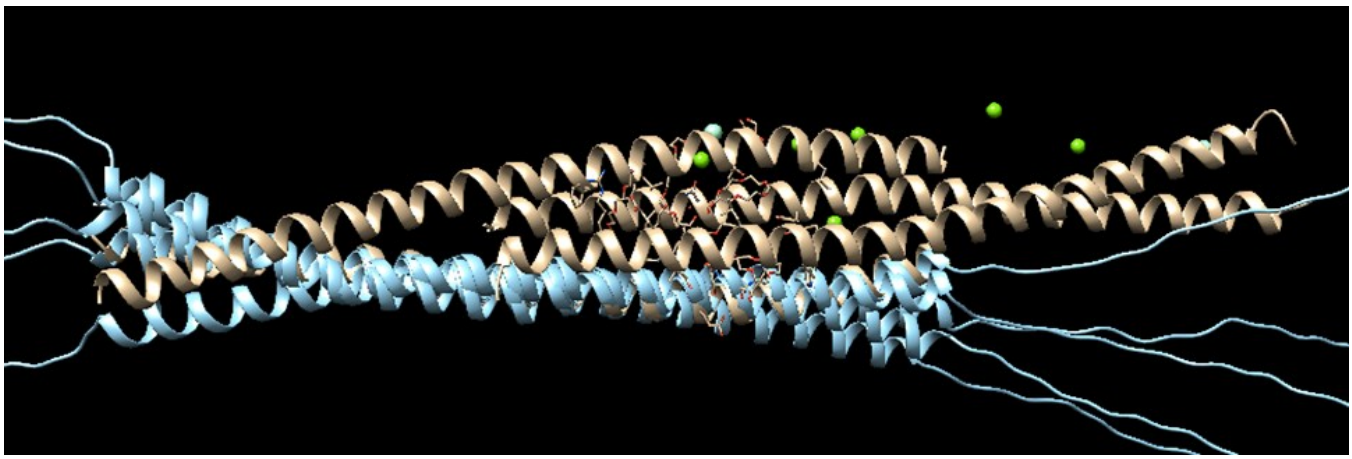

Fig. 4. Conceptual model of GFAP 3D structure (amino acids 65-379) consisting of vimentin (3KLT) backbone (brown alpha helices) superimposed with crystal structures of seven other homologues of GFAP domains (blue alpha helix).

homologues utilizing modeling software Chimera. Using the general framework of GFAP domains as the schematic design, the crystal structure of vimentin was selected as the backbone (brown helices) and additional crystal structures bearing homology to corresponding GFAP subunits (blue helices) were superimposed onto it (Fig. 4). These subunits were selected from a list of candidate crystal structures generated by BLAST search within Chimera based on the sequence identity and the E-value (Table 4).

Discussion

Presence of Coiled Coil Conformation in GFAP

Direct study of GFAP is currently limited due to lack of its reported crystal

Table 2. Identities of amino acids in the core region of GFAP domain 2A coiled coil

Seq. Position	Amino Acid	Heptad Position	CC Probability
231	Leucine	d	0.689
235	Leucine	a	0.689
238	Isoleucine	d	0.689
242	Tyrosine	a	0.689
245	Methionine	d	0.230
250	Methionine	a	0.008

Table 3. Effect of hydrophobicity, size, and polarity of amino acid 239 on the coiled coil formation of GFAP 2A. (L: large, S: small, P: polar, NP: nonpolar, CC: coiled coil, N: no coiled coil)

Mutation from R239 to:	Hydrophobicity	Size	Polarity	Coiled Coil
K, N, D, Q, E	Hydrophillic➞Hydrophillic	L➞L	P➞P	CC
H, T	Hydrophillic➞Neutral	L➞L	P➞P	N
P, G	Hydrophillic➞Neutral	L➞S	P➞NP	N
Y, W	Hydrophillic➞Neutral	L➞L	P➞NP	N
S	Hydrophillic➞Neutral	L➞S	P➞P	N
G	Hydrophillic➞Neutral	L➞S	P➞NP	N
A, F, I	Hydrophillic➞Hydrophobic	L➞S	P➞NP	N
M, L, I	Hydrophillic➞Hydrophobic	L➞L	P➞NP	N
C, V	Hydrophillic➞Hydrophobic	L➞S	P➞P	N

Table 4 Closest homologues of GFAP with known 3D structure listed by BLAST search in Chimera. The homologues were chosen based on regular Sequence identity of GFAP and its homologues

Homologues of Human GFAP	Sequence Identity	E-value	UniProt
HUMAN GFAP	100.00%		
HUMAN VIMENTIN (Coil 2)	62.50%	1e-21	P08670
HUMAN VIMENTIN (Linker1 & Coil 1B)	60.38%	4e-22	P08670
HUMAN VIMENTIN (Coil 1A & 1B)	60.44%	4e-24	P08670
HUMAN VIMENTIN (Coil 1B)	62.79%	4e-22	P08670
HUMAN KERATIN 5,14 (Coil 2B)	42.64%	6e-20	P13647
HUMAN LAMIN-B1 (Coil 2)	44.44%	5e-07	P02545
HUMAN LAMIN (2B)	47.14%	5e-07	P02545

structure. However, we were able to explore important aspects of its structural and functional characteristics by comparative analyses of vimentin, its closest homologue bearing 76% sequence match and with known crystal structure. While some differences could be expected, examination of conserved domain 2A of GFAP in reference to vimentin revealed the presence of alpha helical coiled coil conformation. Coiled coils are found in cytoskeletal, motor, receptor proteins, etc., supporting crucial aspect of their molecular architecture and function[14]. Typically coiled coils specify subunit organization with a left-handed directionality with characteristic heptad repeat units in each participating helix (Fig. 2b). Each of the amino acids in the heptad is assigned a position letter: a, b, c, d, e, f and g. The first amino acid [a] and the fourth amino acid [d] are located internally facing the core of the coiled coil, and are typically hydrophobic residues that form a nonpolar "stripe"[14]. This becomes a prerequisite to form a continuous coil and to serve as a structural backbone. On the other hand, external residues such as [e] and [g] are polar and hydrophilic, reinforcing the adherence between the interacting alpha helices.

Effect of R239C Substitution on the Stability of Coiled Coil

Residues at positions [a] and [d] of GFAP domain 2A were found to be nonpolar amino acids (leucine, isoleucine, tyrosine, and methionine; Table 2) conforming to the heptad model system. These would produce nonpolar inter-helical interactions needed to maintain the integrity of GFAP within the core of the coiled coil. Third, arginine 239 of domain 2A corresponds to heptad

position [e] and is expected to support polar inter-helical interaction possibly with residue at position [g] of domain 2B (i.e., for staggered alignment of chains). Arginine is large, hydrophilic, and polar in characteristics, while cysteine is small, hydrophobic, and polar. Differences in these properties are sufficiently significant to interfere with inter-helical interactions and thus coiled coil formation as is evident in R239C substitution. In turn, the loss of coiled coil could affect the normal kinetics of oligomerization for GFAP. Our predictions are in good accordance with immunoblot analysis that shows, under native conditions, the polymerization profile of wild type and R239C GFAP are different, the former consisting primarily of mono, di, and trimeric forms, while the latter that of tetra, penta, and hexameric forms (GM Tang, unpublished data). It is conceivable that the properties of these heavier oligomers can lead to accumulation of proposed polymerization intermediates[2] that lead to adverse effects manifesting as AxD symptoms.

Conceptual Model of the GFAP Structure

As a concluding note, there is no doubt that a crystal structure of GFAP would add clarity to current understanding of its kinetics. In the meantime, however, conceptual models such as the one presented in this report can still deepen the insight needed to explore the horizon ahead.

Acknowledgments

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Mini-review

Glycemic Control Challenges for Persons with Type 1 Diabetes Trekking at High Altitude

Esther H. Adler and Laura Y. Lorentzen

Kean University, Union, NJ

Abstract

Few studies focus on the effects of blood glucose levels in Type 1 diabetes and how they can affect extreme physical activity at high altitude. This integrative, narrative review of the published literature was searched using electronic databases of English language journals, and covers studies involving Type I diabetics trekking at high and extreme altitudes. We sought to find the commonality of the Type 1 diabetic climber's blood glucose control and their overall athletic success and to refine future research questions. There is some evidence that an increase in insulin when hiking above 5,000 meters, is necessary to maintain normal glucose levels. We conclude that summit success is directly correlated to glucose control and acclimatization. More studies on gender differences in terms of blood glucose metabolism in altitude in persons with diabetes Type 1 are warranted to further analyze how glucose levels are affected by high and extreme altitude.

Introduction

Diabetes mellitus is a prevalent disease that affects blood glucose levels. There are primarily two types of diabetes, commonly referred to as Type 1 and Type 2. Type 2 diabetes (DM2) results from resistance to the insulin that the body produces. The main way to treat DM2 is through diet and medication. In some cases, due to disease progression, insulin therapy is needed. However, this does not mean that a person with DM2 acquires Type 1 diabetes. The prevalence of Type I diabetes is 1 out of 300 in the USA[1]. In Type 1 diabetes (DM1) it is the beta cells, located in the islets of Langerhans of the pancreas which either do not produce enough, or any, insulin. DM1 is hypothesized to be triggered by environmental factors in a susceptible genome. Viral infections may be a possible trigger, but there is uncertainty whether a virus is a sole cause or if it is linked to other factors such as insufficient vitamin D, or a gluten intolerance[2,3]. The only way to treat the disease is by insulin therapy. Without this hormone, blood sugar will continuously increase. There is a relationship between insulin and glucagon, such that glucagon promotes glycogenolysis in the liver and subsequent release of glucose into the blood, thus promoting gluconeogenesis

in the liver and kidneys. The balance between the two hormones is constant, whether one does, or does not consume dietary sugar. Without some amount of insulin in the body, blood sugar can become dangerously high with the immediate risk of ketoacidosis. If untreated or poorly controlled, chronic hyperglycemia can lead to numerous complications including retinopathy, heart disease, gangrene, loss of limbs and blindness[4].

Healthy blood glucose levels range from 72 to 90 mg/dl (4 to 5.5 mmol/L) in the non diabetic population. The fasting plasma glucose threshold for diabetes is 126 mg/dl (7.0 mmol/L)[4]. Anything from 120 mmol/L and higher is considered diabetes. For persons with DM1, maintaining blood sugar between 90 and 150 mg/dl (4.7-8.3 mmol/L) is considered well controlled. People with DM1 face the risk of hypoglycemia, when either taking too much insulin or due to exercise-induced hypoglycemia. Symptoms of hypoglycemia include sweating, hunger and paresthesia, confusion and fatigue[5]. This can be dangerous and sometimes fatal. Recommended blood glucose measurements need to be individualized[6]. Every three months diabetics take a blood glucose test called HbA1c that calculates the average over the three-month period. Non-diabetic ranges are from 4 to 5.5 mmol/L. For the diabetic population, an HbA1c below 7 is recommended, but it is highly individualized, depending on the age, health and physical fitness of the patient[6].

Maintaining normal blood glucose levels for DM1s is imperative in order to minimize the risk of diabetic complications. Leading an active lifestyle is a positive step in maintaining blood glucose levels, but can pose risks for DM1s who experience hyperglycemia during short, intense physical activity (due to the release of counterregulatory hormones) or during moderate continuous activity[6]. More people with DM1 are participating in extreme sports, including mountaineering[7]. However, there are few studies that look at the physiological effects of exercise in DM1s and specifically mountaineering in people with DM1. There is evidence that blood glucose levels increase above 5,000 meters,[8-11] but it is inconclusive if this happens below 5,000 meters and at what altitude the change occurs. It is, therefore, necessary to better understand the relationship between high altitude mountaineering and blood glucose levels in people with DM1.

This integrative, narrative review focuses on the physiological challenges faced by DM1s trekking at high altitude. The scope of the review concerns how high and extreme altitude affects the DM1 climber's blood glucose control and their overall athletic success. In this review of the published literature searched using electronic databases of English language journals, studies involving persons with DM1 trekking at high altitudes were synthesized to find the commonality of concepts and refine future research questions.

Physical Activity and Diabetes

Exercising, although extremely beneficial for persons with diabetes, presents serious challenges. A diabetic can experience both hyperglycemia and hypoglycemia, which can severely disrupt or end a physical workout. Such is a source of frustration to those aiming to live a healthy lifestyle and stay active. It becomes even more complicated to those participating in competitive sports

or extreme athletic activity. Since insufficient insulin is being produced, DM1s, in particular, have a harder time controlling blood sugar, especially during exercise. Either too much insulin can be in the system or not enough, depending on the activity[6]. Robitaille *et al.*[12] found that exercising DM1s rely more on muscle glycogen than blood glucose oxidation in their study of moderate intensity exercise with glucose ingestion. Mohajeri *et al*.[13] suggest a reduction of insulin, prior to exercise, can help the body reduce its reliance on carbohydrates as fuel and use fatty acids during exercise.

Exercise reduces the need for basal insulin, due to its natural non-insulin-dependent glucose transport into skeletal muscle. When exercising at sea level, guidelines suggest that basal insulin should be reduced, as well as a reduction the night after exercise to avoid hypoglycemia[14,15]. Richards and Hillebrandt[15] continue to explain that high intensity exercise can cause hyperglycemia, due to the increase in counter-regulatory hormones. Wheatley *et al*.[16] found that impaired glycemic control lowers the lung membrane diffusion and oxygen saturation during exercise in DM1s. In diabetics that are optimally controlled (HbA1c ≤ 7) they found no difference in lung membrane diffusion rates, compared to non diabetics at rest and during exercise.

Altitude complicates things even more, requiring an additional increase in insulin, attributed to an increased response to the sympathetic nervous system and hormone release as a response. Hormonal changes are seen in climbers without diabetes trekking in altitude. People with diabetes can experience either hyperglycemia due to increase in insulin resistance or hypoglycemia as a result of altitude, which induces anorexia and a delayed absorption of carbohydrates[13]. In a case report by Valleta *et al*.[17], the environment seemed to be the key factor to prolonged hyperglycemia, in a 27 year old DM1 woman trekking at altitude, in a tropical climate. Because the management of blood glucose becomes increasingly more difficult in altitude, adding the extreme physiological stress of climbing in conjunction to altitude adds another degree of complication.

Exercising in altitude creates an additional challenge. In untrained athletes, for example, aerobic capacity (VO_{2max}) decreases 1% for every 100 meters ascent above 1,500 meters[18]. Khodaee *et al*.[7] did a study comparing ultra-endurance athletes with DM1 to those who are not diabetic. They collected data from two different races that took place in Leadville CO, which stands at 3,094 meters (10,151 ft). One race was a 161 km mountain bike race and the other was a 161 km ultra-marathon. Polling both races together, they found 19 out of 7,215 athletes had DM1; all participating DM1s finished the race. While the results were inconclusive due to the small number of DM1s, what they did find was that well-controlled DM1s can participate in extreme athletic activities, including at high altitude, and that more and more DM1s are likely to join these activities[7].

Insulin Challenges at High Altitude

More DM1s are now physically active by making use of insulin pump therapy and continuous glucose monitoring machines. Such technological advances have led to better management during

exercise and the chance for diabetics to take part in a greater variety of extreme sports such as high altitude trekking and mountaineering. Indeed, while older glucose meters (in studies published 1989 through 2001) were known to fail or else not work properly at altitude[9,19-22], certain newer meters have been shown to work with reliability in altitude, while others are influenced by hematocrit[23]. Cold may affect the efficiency of insulin absorption into subcutaneous sites and may pose additional challenges in the correct delivery of insulin, increasing the possibility of hyperglycemia[24]. Zisser *et al.*[25] found that where the traditional insulin pump (relative to its tubing) is placed has a significant effect on delivery. Hence, elevation, temperature and humidity[26] as well as pump type and placement play a key role in the accuracy of glucose meters.

Keys to success when trekking at altitude are glucose monitoring and gradual acclimatization. Such was detailed in a 2001 study by Admetlla *et al.*,[8] where 8 diabetic climbers ascended Aconcagua, located in Mendoza, Argentina, which stands at 6,962 meters (22,841 ft.). Their work suggests that, at high altitude, bolus insulin should instead be taken after a meal as a precaution. Similarly, a 2011 study by de Mol *et al.*,[11] saw 8 subjects with complication-free DM1 climb Mt. Meru, a 4,562 meter (14,968 ft.) peak, followed by climbing Mt. Kilimanjaro located 70 km to the west. Their aim was to investigate insulin requirements, blood glucose levels, energy disturbance and how it relates to acute mountain sickness. The success rate for both expeditions was very high, with six summiting Mt. Meru and all eight summiting Mt. Kilimanjaro. The two individuals that did not make it up Mt. Meru stayed at high camp for reasons unrelated to diabetes. While de Mol *et al.*[11] concluded that insulin requirements increase above 5,000 meters and might be partially due to acute mountain sickness, they were inconclusive as to why.

Amidst reports of insulin requirements varying at altitude, Richards and Hillebrandt[15] performed a detailed review in 2013 of insulin administration at high altitude. Pump therapy and insulin pens are a popular choice, due to the degree of flexibility afforded. Through trial and error, a diabetic can decide which format helps maintain good glucose control. Indeed, a 2017 report[27] of 19 trekkers with DM1 employing modern personal insulin pumps at altitude above 5000 meters, met with success. While there are several ways to administer insulin, achieving glucose control during exercise and at high altitude requires flexibility in insulin dosing and can be the difference between a successful experience and metabolic complications[15].

Metabolism by Gender in Type 1 Diabetics at Altitude

There are many factors to consider when observing the DM1 athlete. For example, there is a marked difference in metabolic oxidation between men and women during exercise. Mauvais-Jarvis,[28] who did a study focusing on gender differences during exercise with healthy adults, discusses how men primarily metabolize glucose during exercise, while women metabolize fatty acids. Estrogen seems to play a role in the metabolic differences. This same observation has been observed for exercise at higher altitude. According to Braun *et al.*,[29] females use less muscle glycogen and/or carbohydrates when there is an increase in catecholamine,

related to the sympathetic nervous system. Men will metabolize glucose, while women instead tend to rely on fatty acids.

Pavan *et al.*[30] performed a study where 6 DM1s and 10 non-diabetics climbed Cho Oyu in China, the sixth highest mountain in the world (8,201 m). Among the 16 climbers, 1 diabetic and 3 non-diabetics summited. Among the six DM1 climbers, one was a female. The DM1 group progressively increased their insulin intake, especially from 3,700 to 4,200 meters and there was a significant increase of basal insulin for the DM1 who summited "from 38 ± 6 units/ day at 0 m to 51 ± 6 at 4,200 m"[30]. They acknowledge that there might be a difference in how men and women metabolize glucose in altitude, which can play a significant role for DM1 female climbers. Of note in their study, blood glucose was controlled and there was no difference in acute mountain sickness among the two groups. The success rate was unrelated to diabetes.

Acute Mountain Sickness in Type 1 Diabetics

Nonacclimatized hikers ascending to high altitude may be at risk of acute mountain sickness (AMS), which can present severe complications for the climber. Altitude poses additional risk for the DM1. Brubaker[31] reviewed several case studies of male and female diabetics climbing in high and extreme altitudes. Brubaker defines high altitude at 3,000 to 5,000 m (10,000 to 16,000 ft.) and extreme altitude above 5,000 m. As one climbs to higher altitudes, air pressure falls. As a result, the partial pressure of oxygen (PO_2) that is breathed in, as well as oxygen saturation in the blood, is reduced. This leads to hyperventilation, which increases PO_2 and activates the sympathetic nervous system[18]. Oxygen supply increases in the body within a few days of being in altitude, and erythropoiesis increases within a few weeks, raising hemoglobin concentrations and improving physical performance[18].

The role of a common drug used in the prevention of AMS, the carbonic anhydrase blocker acetazolamide, was evaluated by a systematic review and meta-analysis. Kayser *et al.*[32] concluded that the drug's efficacy in AMS prevention "is limited when the baseline risk is low." Furthermore, "unless the baseline risk of AMS is high, as with rapid transport to high altitude" the drug "has limited effectiveness"[32]. In this regard, Miller[33] presented a case study of a well-controlled DM1 man who developed AMS as well as hyperglycemia, after flying from sea level to high altitude. The man was treated with acetazolamide. His AMS disappeared, but his hyperglycemia continued while on the drug. Within 24 hours of discontinuing acetazolamide, his blood sugar returned to normal. Miller[33] questioned the safety of acetazolamide for DM1s.

The safety of acetazolamide has been raised for DM1s trekking in extreme altitude. In the Moore et al.[9] Mount Kilimanjaro expedition, 6 out of 15 DM1s summited, while 16 out of 22 non-diabetics summited. The diabetics and most of the non-diabetics were not accustomed to hiking and mountaineering, but underwent some low altitude training in order to prepare. The diabetic group consisted of several persons with preexisting conditions, including retinopathy, microalbuminuria and hypertension. Only one of the non-diabetic participants had pre-existing hypertension and underwent more tests before being allowed to participate. The

climbers were advised to take acetazolamide to prevent or treat AMS and were asked to lower their basal insulin to prevent hypoglycemia, due to the extreme physical activity in altitude. Many of the diabetics who did experience AMS had more severe reactions, as well as hyperglycemia and ketoacidosis. Moore *et al.*[9] support DM1s in pursuing mountaineering and acknowledge that there are many questions, such as how to control AMS and blood glucose levels that still need to be answered.

Controlling blood glucose levels becomes an additional challenge when preventing or dealing with AMS. Mohajeri *et al*.[13] found elevated counter-regulatory hormones can make it more difficult to control blood glucose, especially when the climber experiences AMS. It is unclear if AMS is directly correlated to hyperglycemia or if taking medication (such as acetazolamide) causes uncontrolled hyperglycemia. It is, therefore, important to further study how persons with DM1 can maintain control of blood glucose levels during mountaineering in high and extreme altitude.

Conclusions

How does a person with DM1 control blood glucose levels properly when trekking at high altitude? At what altitude do climbers change their basal rate, from decreasing his/her basal insulin in comparison from sea level, to an increase in his/her basal insulin in comparison from sea level? What kind of adjustments need to be made in basal insulin during rest days at high altitude?

There are only limited studies available, which leaves many questions unanswered. Moore *et al.*[9] asked an additional question related to altitude-induced anorexia. At a certain altitude, a climber can experience altitude-induced anorexia, which appears to exasperate a hyperglycemic incident. If appetite is severely lowered during a climb, does the climber still maintain an elevated basal rate, or will a lower amount of carbohydrate intake require less insulin, even at extremely high altitudes?

As summarized in Table 1, persons with DM1 can succeed in climbing at high altitude by adequately preparing for the unique challenges involved. Indeed, there is some evidence that an increase in insulin when hiking above 5,000 meters, is necessary to maintain normal glucose levels. With knowledge of the effects of high altitude, frequent monitoring of blood glucose levels, and making the necessary adjustments to basal and bolus insulin, a healthy, physically fit DM1 can trek at high altitude and such activity can serve as a healthy way to stay active, as well as increase one's confidence in self-management[13].

A review of the literature suggests that summit success is directly correlated to glucose control and acclimatization. Few studies though focus on the effects of blood glucose levels in DM1s and how it can affect the success of extreme physical activity at high altitude. Furthermore, understanding the difference between male and female climbers, in how they metabolize blood glucose and how hormonal differences play a role in the climber's success has not been sufficiently studied in persons with DM1. Future study to eliminate some of the variables is necessary to further analyze how persons with DM1 can maintain control of their blood glucose levels when trekking in high and extreme altitude.

Table 1. Chronology of Major Studies of Type 1 Diabetics Trekking above 5,000 meters

Study Name	Authors	Description	Results
Management of diabetes at high altitude	Admetlla, Leal and Ricart (2001)	Optimal glucose management, combined with acclimatization can lead to success for diabetics at high altitude	All eight diabetic climbers successfully ascended Aconcagua by managing their glucose and acclimatizing gradually
Extreme altitude mountaineering and type 1 diabetes: the Diabetes Federation of Ireland Kilimanjaro expedition	Moore, Vizzard, Coleman et al. (2001)	Examined the relationship between extreme altitude mountaineering and glycemic control in type 1 diabetes to establish if diabetics are predisposed to acute mountain sickness	Diabetics face significant risks when participating in extreme altitude mountaineering, including glycemic control, ketoacidosis, retinal hemorrhage due to inaccuracies with glucose meters and changes with insulin requirements
Extreme altitude mountaineering and type 1 diabetes: the Cho Oyu alpinists in Alta Quota expedition Metabolic and cardiovascular parameters in type 1 diabetes at extreme altitude	Pavan, Sarto, and Merlo (2003) Pavan, Sarto, Merlo et al. (2004)	An examination of the vital indexes, metabolic control and symptoms of acute mountain sickness during an expedition of Cho Oyu	Well controlled type 1 diabetic patients who are diligent in glucose monitoring and in adjusting dietary and insulin intake can successfully venture into these extreme environments
Increased insulin requirements during exercise at very high altitude in type 1 diabetes	de Mol, de Vries, de Koning et al. (2011)	The relationship between acute mountain sickness and insulin requirements	Insulin requirements increase in altitudes above 5,000 m, during high altitude trekking
Ultra-endurance athletes with type 1 diabetes: Leadville 100 experience	Khodaee, Riederer, VanBaak et al. (2015)	A report of the number and performance of DM1 participants of the Leadville 100 races	There was no statistical difference between the DM1s who finished the race and other athletes
Diabetes, trekking and high altitude: recognizing and preparing for the risks	Mohajeri, Perkins, Brukaber et al. (2015)	A review of key physiological and clinical issues that affect diabetics traveling in altitude	Well controlled and physically fit diabetics, who are prepared for the challenges associated with high altitude trekking can successfully meet their goal

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Two Types of Cells Are Positively Labeled for Heparin in Surviving Organotypic Culture of the Optic Tectum of Adult Zebrafish Brain

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Abstract

The presence of heparin was detected in tissue samples of the zebrafish optic tectum maintained in organotypic culture for up to 7 days. It was found that granules of mast cells with typical morphology were labeled positively for heparin sulfate and at the same time, small, non-granulated cells showed positive labeling for the same heparin sulfates only in their nuclei. The two types of cells and their possible function is discussed.

Introduction

In our earlier work we demonstrated that pieces of the adult zebrafish optic tectum survive at least two weeks in organotypic culture[1]. The surviving tissue shows not only signs of degeneration, but also that certain elements of different regions not only can survive but more importantly form new neuronal tissue, resembling the wall of the embryonic neural tube. We believe that dormant stem cells become injury-reactivated and are responsible for the formation of these new structures. One of our findings was the presence of numerous, large, granulated, cells, which are more densely populated in areas where neuovascularization, hematopoesis and new neuronal tissue formation can be detected. This may indicate the importance of these cells in maintenance of the organotypic culture and neuroregeneration after traumatic brain injury (TBI)[1].

In this present study, we set out to see if the above mentioned large, granulated cells are reactive against a heparin sulfate antibody. We were interested in this molecular component because mast cells, which have the same morphological appearance as those seen in our surviving tissue, are believed to induce regeneration by releasing heparin, among others[2]. Mast cells may be present in several subtypes, some of which are known to contain and release heparin, a molecule associated with inflammation, a process that activates tissue regeneration in general. Other types of mast cells may lack heparin at all[3].

It has been shown that the adult zebrafish brain also contains mast cells[4]. Since heparin content was found to be species and cell type specific we wondered whether mast cells activated in surviving and regenerating adult zebrafish optic tectum tissue samples might influence regeneration through the release of heparin. For this reason,

we used the same organotypic culture techniques to run a time course experiment as seen in Corbo and Fulop, 2017[1]. Because there is controversy as to whether mast cells express heparin, we applied two different techniques of immunohistochemistry, namely peroxidase-anti-peroxidase (PAP) and immunofluorescence (IF) against heparin. Positive labeling would suggest that these large, granular cells, are indeed a sub-type of the mast cell family.

Mast Cells

Mast cells are immune-cells characterized by large and numerous granules that stains metachromatic with basic dyes, such as toluidine blue turning purple or red. They are believed to be similar to or derived from basophiles. It is common to identify mast cells in loose/ areolar connective tissue though they are proven to be present in many other tissue types, including nervous tissue[5]. As we mentioned above, Riley (1956) noted that mast cells are found in high numbers in teleost fish tissues suggesting that in our zebrafish brain tissue samples these cells could be present[6].

Heparin, endogenous molecule in mast cells, is described as a polysaccharide combined from several sulfated disaccharides, which have a relatively large number of linear polydisperse against which antibodies can be obtained[7]. Heparin is a combination of the various and rare disaccharides containing a 3-O-sulfated glucosamine (GlcNS(3S,6S)) or a free amine group ($GlcNH_3^+$). Since there is no uniform structure of heparin, its molecular weight ranges from 3 to 30 kDa[8].

Zebrafish

In the past few decades, zebrafish (*Danio rerio*) became a commonly used laboratory animal with a fully sequenced genome. There is a large amount of literature available about the anatomy, physiology, development and molecular biology of this model animal[4,9-14]. Since it was found that adult zebrafish brain has a high regenerative capacity[15,16], it was a logical selection to use the adult zebrafish brain to study adult brain regeneration after TBI.

Initially, this work was set out to use immunofluorescence technology and the tissue to be analyzed with the confocal laser scanning microscope to detect the presence of the heparin in the organotypic culture of the adult zebrafish brain samples. The benefit of this technology is that relatively thick sections of brain tissue can be analyzed through optical sectioning and three dimensional reconstruction of the tissue sample. However, one drawback to the use of immunofluoresnce in brain tissue in general is the high level of autofluorescnece due to the presence of lectin molecules[17]. This is especially a concern when using such thick samples (greater than 50 μm). For this reason, peroxidase-anti-peroxidase (PAP), detectable as a precipitate under the light microscope, was also used to confirm and support the results found in the confocal laser scanning microscopy.

Materials and Methods

Zebrafish were obtained from Arcadia Pets (Staten Island, NY). Fish were fed daily with a diet of commercial flake fish food. Aquarium conditions were maintained according to the Zebrafish Book[18]. Thirty fish were sacrificed during

this experiment. The usage of animals was approved by The Institutional Animal Care and Use Committee from the New York State Institute for Basic Research (IBR). The number of the animal protocol is 359.

Brain Surgery

Brain surgery was carried out as described by Corbo and Fulop[1]. Briefly, the optic tectum was removed under aseptic conditions and cut into four pieces using a stereomicroscope. The pieces were transferred to the tissue culture facility in pre-warmed culture media.

Maintaining the Organotypic Culture: Conditions and Media

Organotypic cultures were maintained as described in Corbo and Fulop[1] for different time periods: 48 hours, 96 hours, 7 days. Ten fish were used for each time point. The culture media was adopted from Tomizawa *et al*.[19]. At the appropriate point, the tissue pieces were fixed in 4% paraformaldehyde with 0.2% picric acid in phosphate buffer pH 7.2. This is a modified fixative used to preserve antigenicity[20].

General Histology

For general histological analysis, we used Karnowsky's fixative[21], post fixed with osmium tetroxide and processed for semithin serial sections using Spurr resin (Electron Microscopy Sciences), cut with glass knives and stained with toluidine blue as is detailed in Corbo *et al*.[1].

Fluorescent Immunohistochemistry

Tissue pieces were washed in phosphate buffer (pH 7.2) (PB) and extracted in citric acid buffer with a pH of 8.5 and placed in a dry bath incubator for 20 minutes at 80^{O}C and then cooled to room temperature and blocked for four hours 1% saponine in PB solution and 10% fetal calf serum. The primary antibody (goat-anti-heprin, a gift from Dr. Kuberan Balagurunathan, Department of Molecular Medicine, University of Utah) was diluted (1:100) in blocking solution and left on the tissue at 4^{O}C for 48 hours. After incubation, the tissue was washed in 0.1% saponine and the secondary antibody diluted in blocking buffer and left on the tissue for four hours at room temperature protected from light. Sections were washed in PB, mounted on slides and cover slipped with anti-fade mounting medium (1 mg/ml of p-phenylenediamine in phosphate buffered glycerol), for analysis by confocal microscopy. Imaging was done using a Nikon Eclipse E1000 equipped with a Nikon PCM2000 confocal system.

Peroxidase Anti-Peroxidase (PAP) Immunohistochemistry

PAP immunohistochemistry was performed using the protocol adopted from the Vectastain Mouse IgG ABC Kit (Cat # PK-6100). All incubation times were the same as those used for the above procedure. After developing with 3,3'-Diaminobenzidine (DAB), the tissue was washed, dehydrated through an increasing ethanol concentration and embedded in Spurr resin according to the manufacturer's instructions. Tissue was post-fixed with 1% osmium tetroxide prior to dehydration. Sections were cut at 1 μm thick using a Reichert OMU-2 ultramicrotome with glass knives.

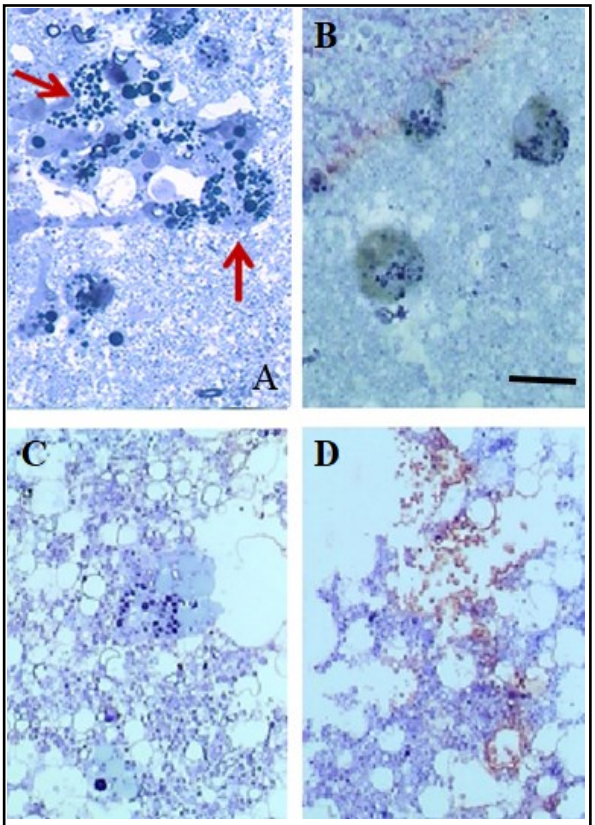

Figure 1 – Microphotographs from toluidine blue labeled 1µm plastic embedded sections. A and B demonstrate large, granulated cells presenting morphological features characteristic of mast cells. Red arrows point to various size granules within the cytoplasm. C and D represents regions which are heavily granulated without the cells being present. In plate D, these granules appear red as a result of metachromasia which is typical for mast cells stained with toluidine blue. Scale bar equals 150 µm

Results

The use of semithin sections (1 µm) stained with toluidine blue is a well-accepted approach to demonstrate the presence of mast cells[22]. In our study, all granulated, large cells mentioned in the introduction showed this characteristic of mast cells. Many of the mast cells appear already degranulated and independent granules are also seen dispersed through the tissue. Figure 1 demonstrates some of these granulated mast cells (A and B) as well as the released granules (C and D) in the surviving tissue samples up to 7-days in culture.

Reactions with the antibody against heparin showed positively labeled cells using both immunofluorescence as well as peroxidase-anti-peroxidase-labeled (PAP) techniques. The labeled cells can be grouped into two categories. In one category, the granules of the large cells, the ones which appeared as mast cells with toluidine blue are antibody-labeled. The second group of cells are smaller, non-granulated and only the nuclei of these cells are labeled positively. Figure 2B demonstrates, in a 48-hour surviving sample, an immunofluorescently labeled granulated cell showing a similar morphological appearance as the mast cell seen in toluidine blue stained semithin sections (Figure 2A). Note that the fluorescent label is specific only to the granules of the cell while the nucleus remained unlabeled. Similarly, granule-specific label was detected using the PAP immunohistochemical method (Figure 3A,B). Both immunohistochemical techniques labeled cells positive for heparin in the nuclei of the second category cells, while the cytoplasm remained unstained (Figures 2C, 3C-F).

Analyzing the tissue in the confocal microscope allowed more labeled cells to be visualized because of the increased thickness of the sample and maximum projection of the collected image stack while the semithin sections labeled with PAP showed better the fine nuclear structures. Similar labeling and distribution of cells was detected in preparations surviving at 96 hours and 7 days.

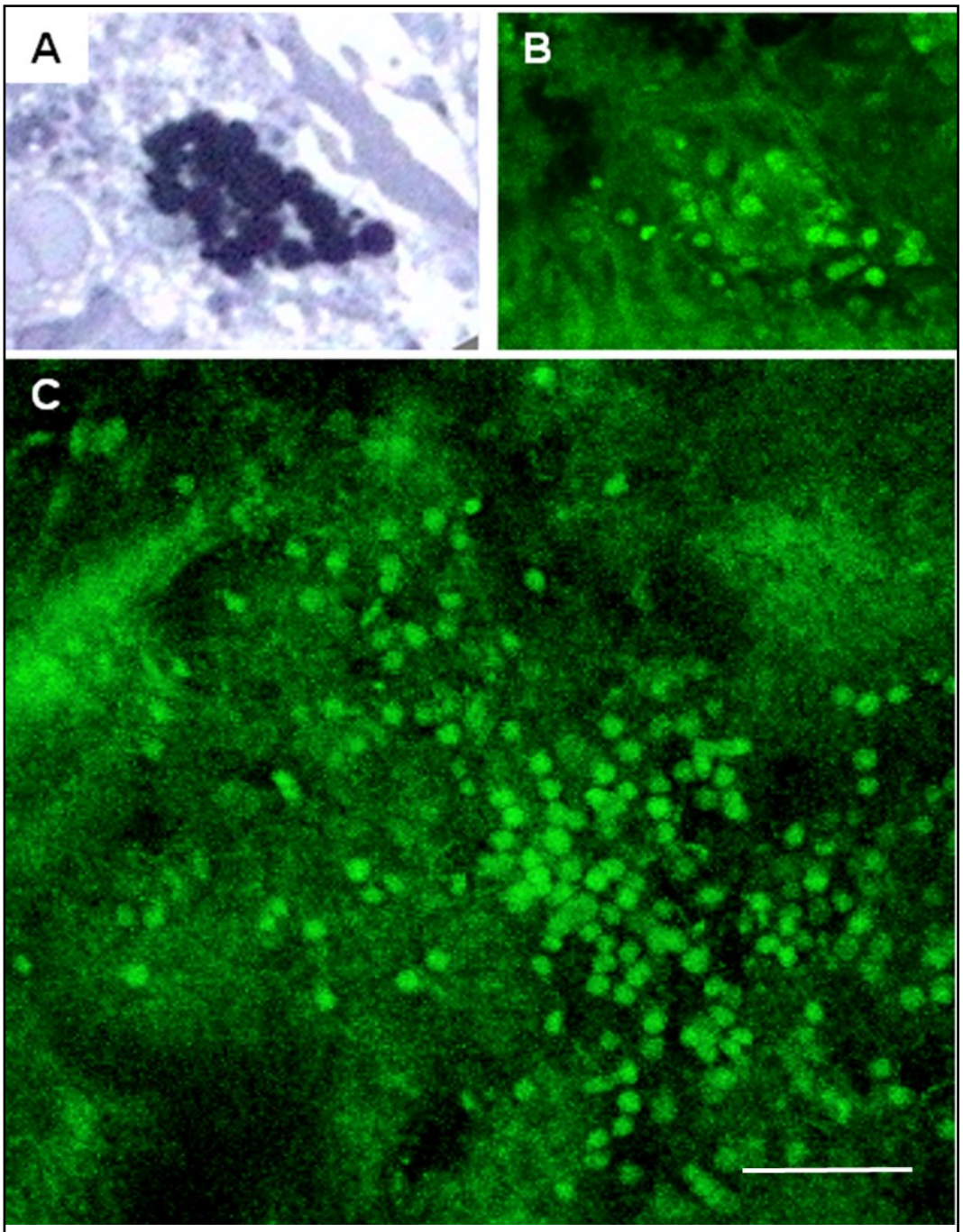

Figure 2 – Toluidine blue and fluorescent immunolabeling for heparin in surviving optic tectum (7 days). (A) A mast cell as it appears in semithin sections stained with toluidine blue to be compared with (B), that demonstrates the presence of heparin in the granules of similar cells as seen in the fluorescent microscope labeled against heparin with IHC. (C) Are the smaller of the two cell types, with heparin labeling appearing only in the nuclei of these cells. Both (B) and (C) were captured with a 60x oil immersion objective, both are maximum projections of an image stack. Scale bar equals 200 μm.

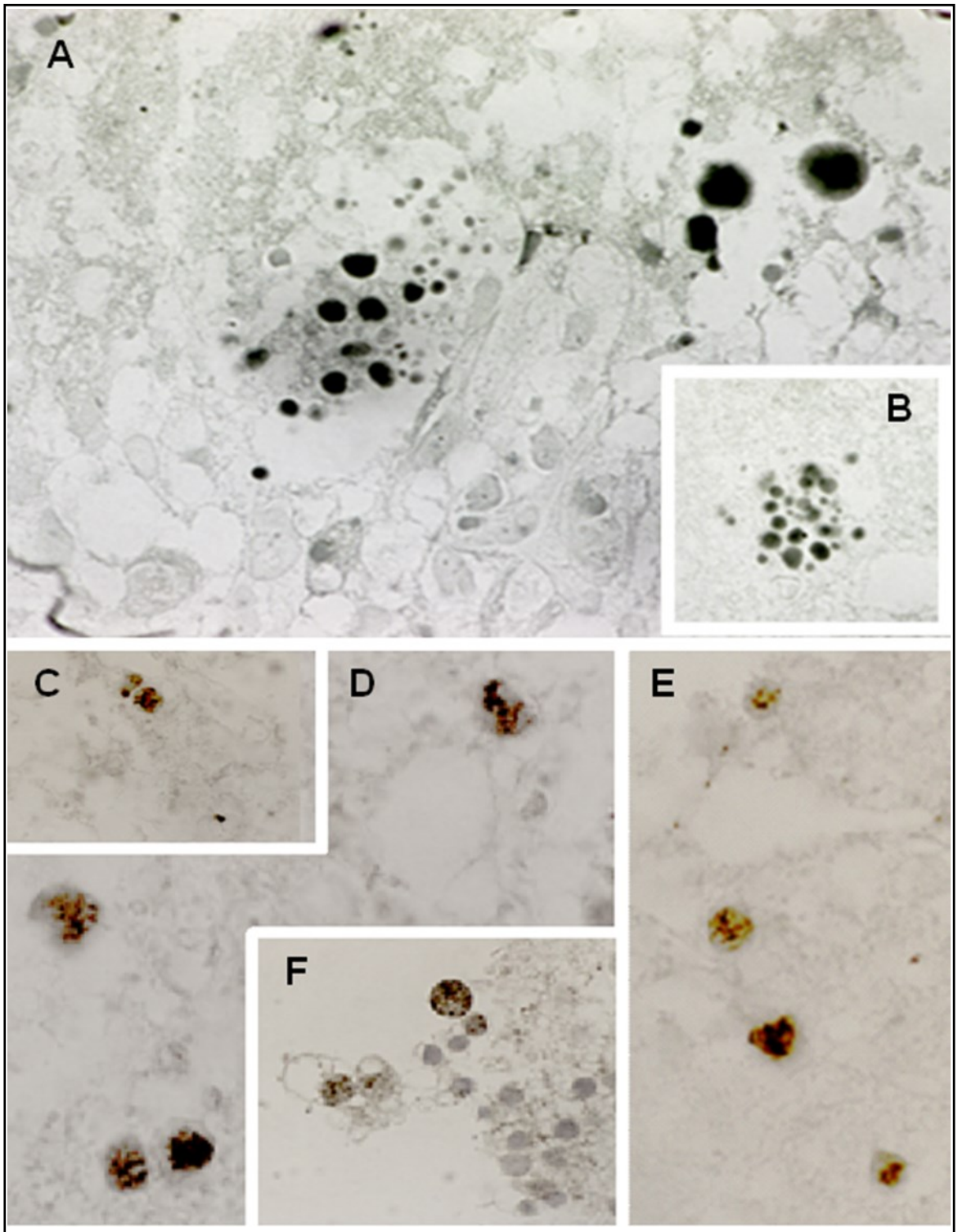

Figure 3 – Light microscopic images of peroxidase-anti-peroxidase (PAP) immunohistochemistry labeling of heparin in seven day surviving organotypic culture samples. (A & B) are images of heparin-positive granules in mast cells. (C – F) Heparin-positive cells in different regions of the surviving tissue piece. Scale bar equals 200 μm.

Discussion

Heparin is a known mediator for many cellular events, including proliferation and differentiation. For example, during embryogenesis in zebrafish, heparin-binding neurotrophic factor was found responsible for axon outgrowth[23].Since heparin has shown developmental properties in an embryo, there is a likelihood that heparin participates in the neuronal regeneration in adults as well. Today, there are studies suggesting that heparin may indeed be a regenerative agent in different tissues of adult vertebrates' brain [23-26]. In every time point, we have found numerous, large granulated cells showing toluidine blue metachromasia typical for mast cells within the organotypic culture of the adult zebrafish optic tectum, our model of traumatic brain injury[1]. Mast cells are known to contain and release heparin among many other paracrine agents[26]. Accordingly, we were interested to see if the cells present in our model indeed would be labeled positively for heparin.

In this study, we analyzed surviving organotypic cultures at 48 hours, 96 hours and 7 days. At each time point, we detected two types of heparin positive cells; large cells in which some of the granules were positively labeled and smaller cells in which only the nucleus showed positive labeling for heparin. We do believe that the large granulated heparin-positive cells are indeed the mast cells. We also noticed that a large number of smaller, non-granulated cells were also positively labeled for heparin, but only in their nuclei. This phenomenon is not unusual. According to Hildebrand and colleagues (1977), when heparin is found binding to DNA in isolated eukaryotic cell nuclei structural and functional alterations are made on chromatin[27,28]. Specifically, the heparin interacts with histones in the nucleus. Using Chinese hamster cells, Hildebrand's group was able to prove that the negative charge on the heparin is able to interact with the positively charged histones, which results in the removal of histones from the DNA[27]. With the removal or alteration in histones, heparin may be responsible for induced change in chromatin organization and, as a consequence, cells may be reprogrammed to have a different fate. Further studies also suggest that heparin interacts with the inner histones (H2A, H2B, H3 and H4) of chromatin and provides cell cycle specific changes during interphase[28].

We believe that these cells with heparin positive nuclei, could be the reactivated stem cells which may, somehow, control the function of the mast cells and induce or inhibit the release of the different paracrine agents including heparin. Inversely, they may be activated by release of heparin by the mast cells. This may open a new avenue to understand central nervous system regeneration in lower vertebrates following traumatic brain injury.

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