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Article

The effect of Omega-3 on bovine blood cells as a potential remedy for Cerebral Cavernous Malformations

Medha Pulluru[1]*, Esha Venkat[2]*, Lahari Vallamkonda[3], Lilian Chen[4], Jessica M. Ross PhD[4]

[1] Vista Ridge High School, Cedar Park, Texas
[2] Westwood High School, Austin, Texas
[3] University of Colorado Denver, Denver, Colorado
[4] Stanford University, Palo Alto, California
* These authors made equal contributions

SUMMARY

Cerebral cavernous malformations (CCM) are lesions on the brain that occur due to a genetic mutation causing high membrane permeability between endothelial cell junctions. They can cause a myriad of symptoms including seizures and aneurysms. Current treatment plans include monitoring for symptoms, tracking disease progression, and surgical correction of malformations, but therapies that target the underlying changes in cell junctions are lacking. The purpose of the study was to investigate whether dietary Omega-3 fatty acids can strengthen compromised cell junctions. The hypothesis was that Omega-3 fatty acids would strengthen the compromised cell membranes due to their high lipid content, increasing membrane thickness. The cow in the treatment group was fed an Omega-3 diet, whereas a cow in the control group was fed the standard corn gluten meal. Blood was then drawn from each of the cows to use in the subsequent studies. Using a microscope, membrane rigidity of whole bovine blood (all blood cell types) was viewed. The whole blood cells were placed in hypertonic solutions as well as isotonic solutions, altering the size of the cells. Control tests on untreated, bovine blood from the corn gluten meal diet in both hypertonic and isotonic solutions were performed for comparison. In support of the hypothesis, the addition of Omega-3 in the bovine diet resulted in an increase in the cell membrane thickness when placed in hypertonic solutions, indicating decreased membrane rigidity. Based on these results, the use of dietary Omega-3 as a possible treatment for CCM should be explored in the future.

INTRODUCTION

A cerebral cavernous malformation (CCM), also known as cavernous angioma or cavernoma, is a vascular malformation characterized by very slow blood circulation in capillary vessels (1). CCMs are prevalent in the children's age group (0.5% of the general population is affected), particularly in a hereditary form called Familial Cerebral Cavernous Malformations (FCCM) (2). FCCM is autosomal dominant, so children have a 50% chance of inheritance if one parent has FCCM (2). One of the main indicators of FCCM is if the magnetic resonance imaging (MRI) of the adult patient shows multiple lesions in the brain, which offers a strong correlation between age and the number lesions (3). Roughly 20% of affected people have a familial (inherited) form of the disorder (3). In many cases, such people can identify similarly affected family members, most often with multiple malformations, which can only be truly verified through genome or genetic testing.

CCM malformations are formed from a deletion mutation in one of the CCM genes (*CCM-1*, *CCM-2*, and *CCM-3*) that causes the loss of the KRIT1 protein. This protein maintains the structural integrity of the endothelial cell junctions, and the lack of the protein leads to the cell junctions weakening and the blood vessels bursting in the brain, which can lead to hemorrhages, seizures, neurologic deficits, and non-specific headaches (4). Seizures and hemorrhages are the most common symptoms of CCM (4).

Current treatments focus on easing the side effects of CCM but do not address the root cause of these malformations. Treatments are determined based on the severity of the malformations. When patients are asymptomatic, doctors may decide to keep the patient under observation. Observation can require intermittent MRI to watch for changes in the malformation. Techniques such as functional MRI and tractography may also be used to monitor functional and structural changes (5). If a patient is experiencing seizures, a medication may be prescribed to decrease seizure frequency. With increased severity of CCM symptoms, a surgical approach may be recommended, in which the whole malformation is removed (6). Our study aims to address the underlying cause of this disease: the compromised cell junctions. To model these conditions, two types of bovine blood cells were used, one extracted from a cow supplemented with increased Omega-3 fatty acids in the diet, and one fed a regular corn gluten meal diet that did not contain Omega-3 (7). Omega-3 was the chosen fatty acid of study because of its unique properties. For example, it's incorporated in every cellular membrane in various tissues and contributes to the quality of life while reducing premature death (8). Unlike other fatty acids, there are many sources of Omega-3 that can be incorporated into a healthy diet such as fish sources, beans, eggs, and flaxseed; fish meal was used in this study (9). Depending on the diet of the organism, the amounts of Omega-3 fatty acids in each of the cell membranes can differ based on intake. Omega-3 has multitudes of other uses in the cell, such as regulating blood clotting, inflammation, and artery wall movement (8). Necessary for generating cell energy, Omega-3 aids the cell in carrying out normal processes (10).

We hypothesized that the addition of Omega-3 would combat the effects of the increasing salinity of the solutions by decreasing the membrane rigidity of the cells. We hypothesized that the bovine blood cells from the Omega-3 diet would not shrivel to the same extent as the blood cells from the corn gluten meal diet in hypertonic solutions, showing that the bovine blood cells from the Omega-3 diet have increased membrane rigidity. In our experiment, the cows were either fed a fish diet (fish have high Omega-3 counts) or a diet of corn gluten meal for 20 days. Blood samples from both the experimental and control groups were collected and placed in different concentrations (0.9 g, 1 g, 1.5 g, 2 g) of sodium chloride solutions. The hypertonic solutions model the mutated CCM cells as CCM cells display similar characteristics of decreased cell rigidity and weakened cell membrane structure as seen in hypertonic solutions. In CCM cells these characteristics occur because of unregulated actin fiber activation (11). In hypertonic solutions, cells weaken because of exosmosis, which causes plasmolysis (12). The corn gluten meal, which we used as the control treatment, has high concentrations of iron, folate, and vitamins but not Omega-3 (13). The addition of the Omega-3 into the diet should decrease the membrane rigidity between the cell junctions of the blood cells due to an increased thickness of the cell membrane, making up for the compromised structure (increased distance between cells) of the cell junction due to the mutation. The increased cell membrane thickness decreases permeability through greater structural integrity, which in turn, decreases the likelihood of the cells lysing, a symptom of CCM (14). The results were analyzed through both quantitative (statistical tests) and qualitative (comparison of cell structure and size) tests, which both showed that Omega-3 will decrease the membrane permeability of the cells.

RESULTS

The cows were either fed a fish meal or a normal corn gluten meal, with the fish meal containing rich amounts of Omega-3. The samples were extracted after 20 days of supplementation and placed in potassium salt vacutainers. The blood samples were then placed in isotonic and hypertonic solutions (the isotonic solution had 0.9 g sodium chloride per 100 mL of water, and there are 1 g, 1.5 g, and 2 g of sodium chloride per 100 mL of water for the hypertonic solutions.) To investigate whether Omega-3 would influence the membranes of red blood cells, we observed the cells from both experimental and control groups under an inverted microscope and quantified the cell diameters. We observed that the size of the cells was visibly different between the bovine blood cells

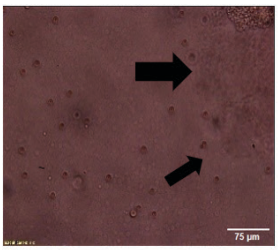
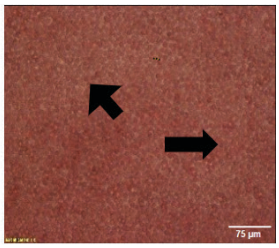
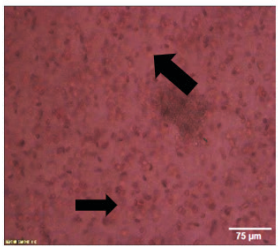
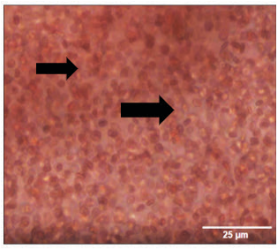
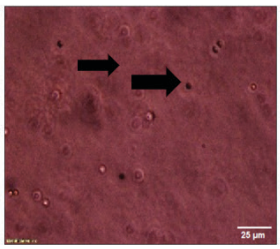
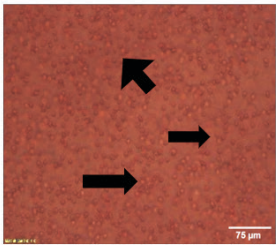
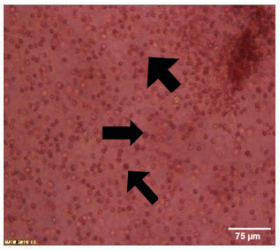
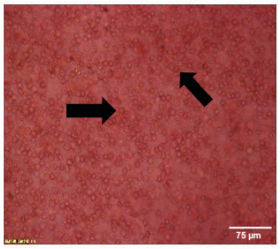
Type of Solution	Isotonic solution: 0.9 g sodium chloride	Hypertonic solution 1: 1 g sodium chloride	Hypertonic solution 2: 1.5 g sodium chloride	Hypertonic solution 3: 2 g sodium chloride
Bovine blood sample from corn meal diet	10x magnification 	10x magnification 	10x magnification 	20x magnification 
Bovine blood sample from the Omega-3 diet	20x magnification 	10x magnification 	10x magnification 	10x magnification 

Table 1: Images of red blood cells in solutions with different sodium chloride concentrations. The first row shows the bovine blood cells from the corn gluten meal diet, and the second row depicts the bovine blood cells from the Omega-3 diet. Qualitative differences were seen in the size and shape between the cells of the different diets and different sodium chloride concentrations; bovine blood cells from the Omega-3 diet displayed an overall round and smooth-edged structure throughout the saline concentrations. For the images at a 20x magnification, the scale bars read 25 µm, while the images at 10x magnification are presented with a 75 µm scale bar. The arrows on the images are referring to individual cells where the contrast in cell size per solution and blood type is most apparent visually.

from the Omega-3 diet and corn gluten meal and across solution types. As the salinity concentration increased across the solutions, the bovine blood cells from the Omega-3 diet maintained a relatively larger circular shape in comparison to the bovine blood from the corn gluten meal diet. Moreover, the bovine blood from the corn gluten meal diet visibly shrunk and transformed into a more oval-like shape over the increasing salinity concentrations **(Table 1)**. Since the increasing salinity aims to model CCM, the Omega-3 diet demonstrates how it could potentially help maintain cell structure even when CCM is damaging it.

Hypertonic solutions resulted in more shrinkage due to the increased sodium chloride concentration. The isotonic solution had the least effect on cell size, as the diameters of the cells remained rather large compared to the hypertonic solutions **(Figure 1)**. The diameters of the bovine blood cells from the Omega-3 diet were significantly larger than the bovine blood cells from the corn gluten meal diet in all the hypertonic solutions. The cells from the Omega-3 diet maintained their circular shape and structural integrity far more than the cells from the corn gluten meal **(Figure 1)**.

Every *t*-test showed a statistical relevance with a *p*-value less than 0.05, except for the isotonic solution at 0.9 g/100 mL, which further validated our results because the isotonic solution served as our control group when increasing the salinity of the solutions. These results showed a significant change between bovine blood cells from the Omega-3 diet and the corn gluten meal, and since the *p*-values for the hypertonic solutions at 1 g, 1.5 g, and 2 g/100 mL were between 0.01 and 0.001, these concluded strong evidence for greater diameters in the bovine blood cells from the Omega-3 diet as opposed to the ones from the corn gluten meal. These statistically significant results demonstrated how an Omega-3 diet could be a viable remedy for CCM symptoms.

DISCUSSION

The goal of our study was to discern whether Omega-3 would be promising in combating the effects of hypertonic solutions on red blood cells. We tested two different bovine blood solutions; one fed an Omega-3 diet and the other a corn gluten meal. We utilized four solutions (0.9 g, 1 g, 1.5 g, and 2 g of sodium chloride per 100 mL of water) and placed the blood under a photographic microscope, analyzing the images with ImageJ. We found visible differences in the photographs taken with the light microscope. Specifically, the diameter of the cells was significantly larger in the sample containing the

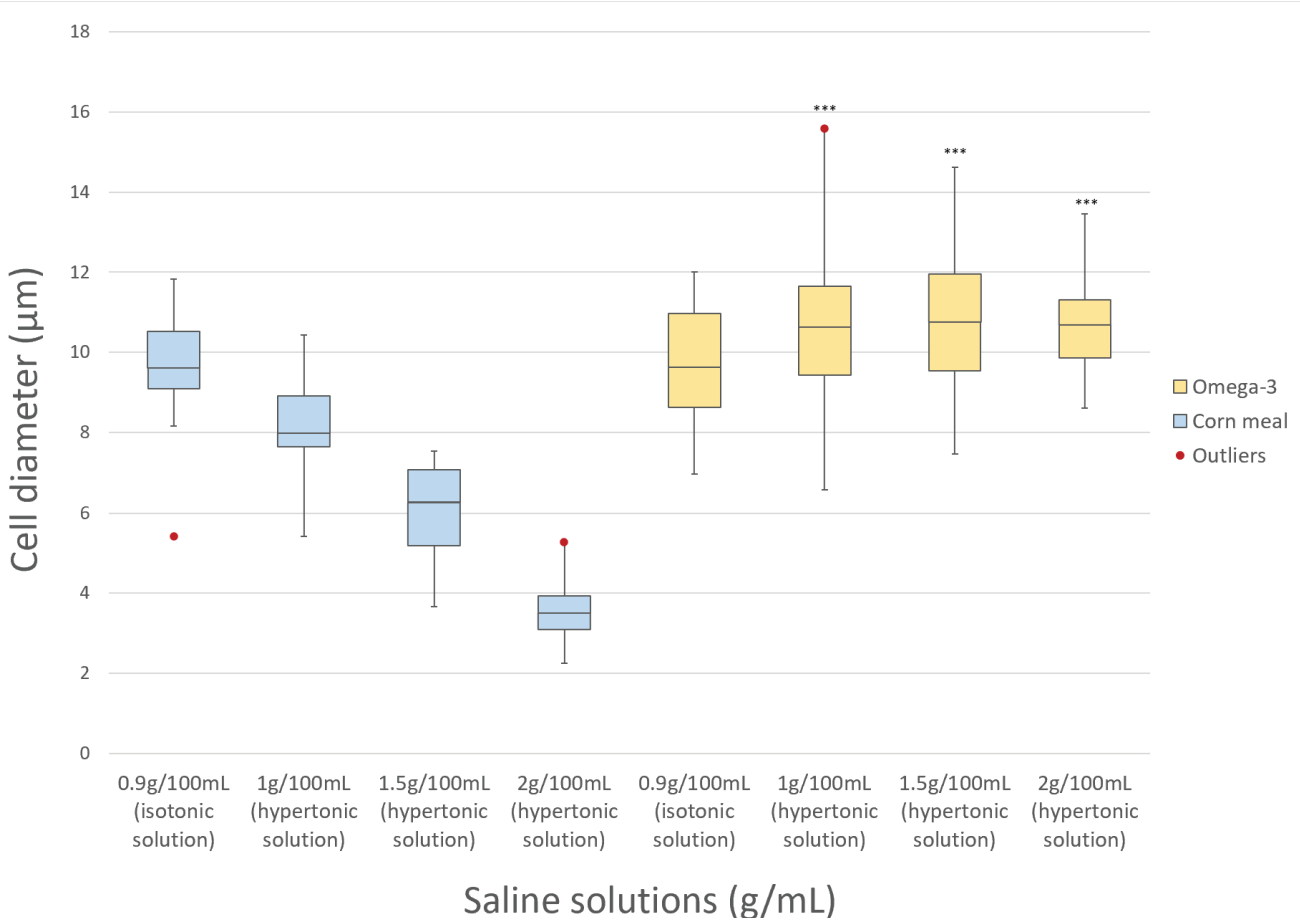


Figure 1: Diameter of blood cells from cows fed corn meal or Omega-3 diets in different sodium chloride concentration solutions. Box and whisker chart comparing the diameter size between the cells in the isotonic and hypertonic solutions (n = 20). The control group was the bovine blood cells from the corn meal diet whereas the bovine blood cells from the experimental group were from the Omega-3 diet. Error bars represent standard deviation, and median lines are present. $p < 0.05$ is shown as ***. There was no significance between the cells from the different diets in the isotonic solution. (0.9 g/100 mL) The bovine blood cells from the Omega-3 diet in the hypertonic solutions (1 g/100 mL, 1.5 g/100 mL and 2 g/100 mL) were significant compared to the bovine blood cells from the corn gluten meal.

bovine blood cells from the Omega-3 diet; the overall size of the cells was less shrunken and appeared less wrinkled in this sample. These differences were most noticeable in the higher concentrations of the hypertonic solution. Supporting this observation, the quantitative measurements taken by ImageJ showed reduced cell diameter for the bovine blood cells from the corn gluten meal diet in comparison to the Omega-3 diet. In every concentration of sodium chloride to water except the 1 g of sodium chloride/100 mL solution, the bovine blood cells from the corn gluten meal were smaller than the bovine blood sample from the Omega-3 diet group, both qualitatively and quantitatively. Supported by previous literature, we attributed changes in cell surface area to changes in membrane thickness (15). Our hypothesis was confirmed by these results because the cell surface area increased in the sample with the bovine blood cells from the Omega-3 diet, suggesting that membrane rigidity had decreased. The use of Omega-3 fatty acids could be a promising frontier for future exploration in managing the symptoms of CCM by increasing the surface area of the cell membrane, thereby likely decreasing membrane rigidity of cellular junctions.

A number of limitations in our study should be addressed. Bovine blood cells were used instead of endothelial cells, which are the cell type affected by CCM. Endothelial cells are the cells that line the blood vessels and make up one of the layers of the vessel (16). We chose to use bovine blood cells instead of endothelial cells because access to endothelial cells was prohibitive due to the scope of the study. However, blood cells collected from a local farm were accessible and feasible to use because they could be collected fresh and immediately analyzed for the current study. Our lab was affiliated with the farm, so the condition and health of the cows was known to be typically healthy. Future experiments are needed to investigate the effects of dietary Omega-3 on endothelial cells.

Our quantitative results were limited by a few factors. First, with our limited resources, we measured cell size instead of membrane thickness. Membrane thickness was not directly measured. The results were also limited based on the functionality of ImageJ. Future work should attempt to use more sensitive instruments to assess changes in cell size and characteristics such as 3D optical microscopes. More dimensions should be quantified such as normalized radius, cell texture, and shape. To accurately measure cell membrane thickness, molecular dynamics simulations would need to be conducted. Additionally, to increase the experiment's level of replication, we would like to test blood samples from 20 different cows for each saline solution. The present results suggest there may be diet-based preventative measures that could prove useful for combating compromised cell junctions for patients with CCM. Due to improved membrane rigidity and protecting cells from shrinkage in hypertonic solutions, this research suggests that Omega-3 may also have decreased membrane permeability of bovine blood cells. Omega-3 shows promise as a safe and affordable method for reducing risks associated with these malformations.

Omega-3 is a complex compound that consists of fatty acids, present in many dietary foods such as fish. The sample cows were fed fish meal that consisted of high levels of Omega-3. Other fats could have the same effect on the membrane rigidity of the bovine blood cells because they increase the thickness of the cell-membrane with increased amounts of lipids. A number of other fats that could be tested for maximum effectiveness in future studies about CCM are steroids, chicken fat, different types of oil like olive, sunflower, and soybean oils, or various dairy products.

Our bovine model should be tested using human endothelial cells because these are the cells that make up the majority of vessels where these malformations occur. This same method could be applied to various malformations that happen in the human body, such as arteriovenous malformations but would need to be tested in vivo like CCMs. Because the various side effects of CCM can lead to premature death, preventative diets could have a great impact on an at-risk population (2). As there are no current treatments that address the underlying cause of CCM, this study shows that Omega-3 may prove helpful in preventing the lysing of cells in saline solutions, which models blood vessels bursting. People with the CCM gene, including families, would benefit from these improved health outcomes.

MATERIALS AND METHODS

We chose to use a lab-based experiment to answer our research question, and our methods were split into four parts: sample preparation, solution preparation, bloodwork, and organization of results.

Sample Preparation

On a ranch in Northern Colorado, two non-lactating Angus cows (*Bos taurus*) between the ages of 2–8 years were fed two different diets. One of the cows was fed fish meal that consisted of a 5% dry matter intake (DMI). The other cow was fed a regular corn gluten meal, which consists of a 6% DMI. Both diets were provided by the ranch as part of their usual dietary products. The blood samples were collected from both cows after 20 days of their assigned diet. Immediately after extraction, the blood was placed in potassium salt vacutainers, which prevent clotting. Clotting is known to invalidate the readings and experimentation on freshly-drawn blood (17).

Solution Preparation

Four solutions were created in total, one isotonic solution and three hypertonic solutions, each with different concentrations of sodium chloride. The isotonic solution served as a control solution. Each concentration of sodium chloride was an independent variable to test how the concentration affected the permeability of the cell membranes. We prepared four solutions of various concentrations of sodium chloride per 100 mL of distilled water (0.9 g/100 mL, 1 g/100 mL, 1.5 g/100 mL, and 2 g/100 mL).

Bloodwork

Micropipettes were used to measure 1000 µL of each solution per sample, starting with 0.9 g of sodium chloride. After dispensing the solutions, we used new micropipette tips to measure out 20 µL of the blood type used (Omega-3 diet and corn gluten meal). Then, we dropped the blood into each solution, one at a time, and then immediately started a timer. The timer was used until observation under a microscope for the sole purpose of ensuring each sample had equal amount of time (90 seconds) in the blood to view accurate cell change. We used an inverted fluorescent microscope (Olympus CKX41) under 10x and 20x magnification. Lastly,

we transferred the well plate underneath the microscope after the blood was dropped in each sample to view the visible changes in the cell membrane and document them with pictures.

Data Analysis

We captured photographs of the bovine blood cells from each sample (Omega-3 diet and corn gluten meal) and with each solution. Isotonic solutions and three different concentrations of hypertonic solutions with increasing degrees of sodium chloride were measured. The bovine blood cells from the Omega-3 diet and the corn gluten meal diet were used in each solution, creating a total of eight samples. We analyzed diameters of 20 cells (randomly selected through the blood sample) per 8 samples (in micrometers) with the ImageJ software. ImageJ was used to convert the images to binary, and then each cell diameter was automatically measured by the software. The distance between the two furthest points on the cell's circumference were measured for each cell. Pixels were converted to micrometers, and averages were calculated.

Four *t*-tests were conducted, one for each sodium chloride solution. The *t*-tests were two-tailed and compared the diameters of the cells between the bovine blood cells from the corn gluten meal and the Omega-3 diet group in each solution. The alpha value used to determine significance for the *t*-test was 0.05.

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