Original Article

Cell Count and Cell Density Decrease as Age Increases in Cadaveric Pediatric Medial Menisci

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Purpose: To examine the histologic changes in terms of cellularity, cell density, and nuclear shape in medial meniscal cellularity during skeletal development using pediatric cadaver specimens. **Methods:** Medial menisci from 26 pediatric cadavers, 11 female and 15 male (total 36 menisci), were obtained from tissue bank. Mean age of female donors was 34 months (1-108 months) and of male donors was 52 months (1-132 months). Menisci were processed and embedded in paraffin blocks. Each tissue block containing 6 representative areas of meniscus (anterior root, anterior horn, body [n = 2], posterior horn, and posterior root) was sectioned at 4 microns and stained with hematoxylin and eosin for evaluation of chondrocyte nuclei. Each of the 6 representative areas was imaged at 10×; one image on peripheral one-third of section, the second image on central two-thirds of the section. FIJI imaging software was used to measure cell count, cell density, and nuclear morphology (1 = perfect circle). Data analysis included linear mixed models, Type II analysis of variance tests, and pairwise tests with the Tukey correction to assess statistical significance. **Results:** Peripheral meniscus was more cellular than central meniscus. The cell count was found to decrease by 14% per year of age. Peripheral cell count decreased at a rate similar to the cell count in the central meniscus. Meniscal cell density was 2× higher peripherally than centrally. Overall average cell density in all locations in the menisci decreased by an average of 14% per year of age. **Conclusions:** The results of this study reveal decreases in cell count, cell density, and circularity as age increases in cadaveric pediatric medial menisci. **Clinical Relevance:** To better understand the development of pediatric menisci at a cellular level and use this knowledge in the future on how to maintain the menisci in a younger, healthier state.

The human menisci have an essential role in the biomechanical function of the knee joint. Menisci are primarily composed of fibrocartilage, produced and maintained by chondrocytes.[1-5] The structural components of the meniscus develop over time from the neonate to adulthood, during which there is an increase in weight-bearing.[2] Previous studies on changes in meniscal composition with age have focused on the macroscopic and microscopic changes in the adult menisci during the development of diseases such as osteoarthritis.[3,6-14] Such studies have identified a decrease in chondrocyte cell density and changes in chondrocyte nuclear morphology in the aging meniscus.[12,15-17] Comparable studies on the changes in

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human meniscal cellularity during skeletal development with pediatric menisci, to our knowledge, are limited. The authors of one study commented on the histology of postnatal specimens, but the study lacked quantitative data and did not directly compare structural components in different locations within the meniscus. The quantitative knowledge of menisci cellularity, cell density, and nuclear shape in pediatric specimen is poorly understood.[18]

Awareness of how the healthy human meniscus normally develops and understanding of the progression of the chondrocyte throughout pediatric development may help us further understand how the meniscus changes with age. In the future, better understanding of the developing meniscus may give us insight into how to maintain the meniscus in a younger, healthier state. Proper meniscal biologic state and morphology is critical to maintain during growth in order to ensure a durable meniscus throughout life. This has clinical implications for meniscus repair techniques in skeletally immature patients.[19] In the age of advancing orthobiologics, understanding how the meniscus develops is important for biologic applications.[20-23] Three-dimensional printing of human tissues, including the meniscus, is not far in the future; the development and composition of the meniscus, including cell number, density, and cell development must be understood in order to do that properly.[24-27]

The purpose of this study was to examine the histologic changes in terms of cellularity, cell density, and nuclear shape in medial meniscal cellularity during skeletal development using pediatric cadaver specimens. We hypothesized that the cellularity of the menisci would decrease during development and that changes in cellularity during growth would be influenced by location in the meniscus.

Methods

Approval for this work from our institutional review board was not needed because the specimens that were studied were deidentified knees from human cadavers procured from a tissue bank (AlloSource, Centennial, CO). The medial menisci from 26 cadaveric donors, 11 female and 15 male, were collected, providing a total of 36 specimens (right and left menisci). The mean age of the female donors was 34 months with a range of 1-108 months. The mean age of the male donors was 52 months with a range of 1-132 months (Fig 1).

The menisci were stored frozen at −20°C. After thawing, they were fixed in 10% neutral buffered formalin for a minimum of 48 hours before transfer to 70% ethanol. The specimens were trimmed using a scalpel to remove adjacent soft tissues and were placed intact into appropriately labeled cassettes for routine processing. The processed menisci were cut into 6 pieces and embedded in paraffin in a standardized fashion (Fig 2). The locations of the sections were determined based on gross anatomy by direct visualization of the menisci.

Each tissue block containing 6 representative areas of meniscus (anterior root, anterior horn, body [n = 2], posterior horn, and posterior root) was sectioned at 4 microns and stained with hematoxylin and eosin for evaluation of chondrocyte nuclei (Fig 3).

Each section was imaged using a Nikon Eclipse microscope at 10× (field of view 20) with a Nikon-DS-Ri2

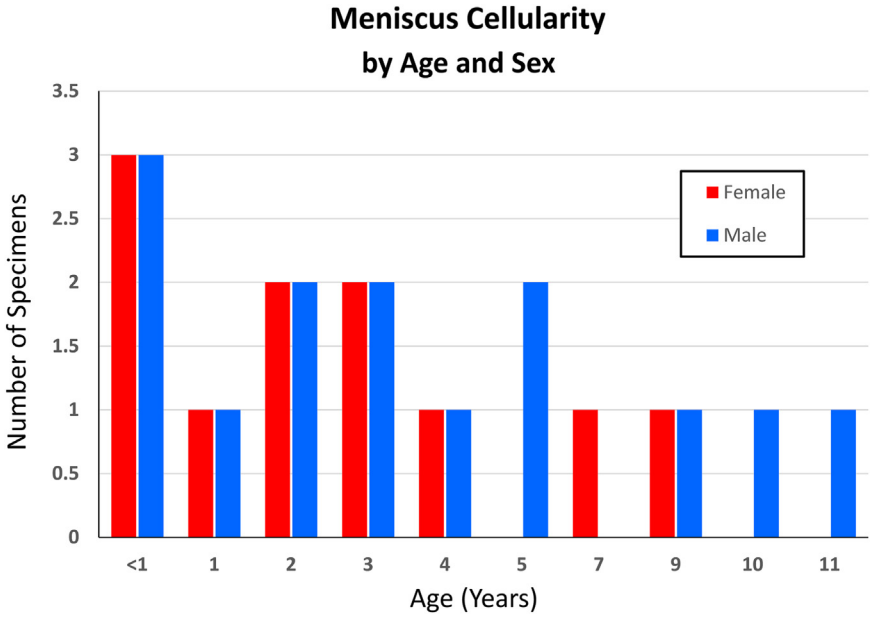


Fig 1. Bar graph demonstrates the distribution of cadaveric ages for female and male specimens.

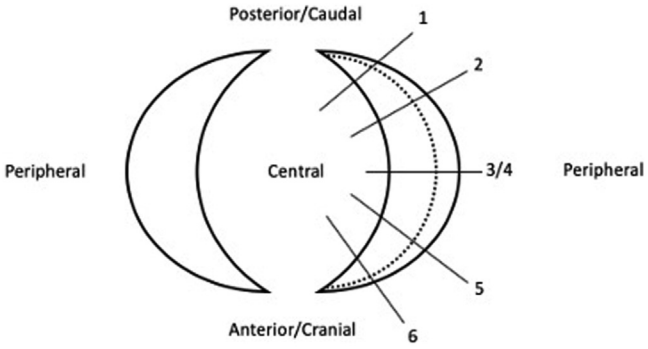
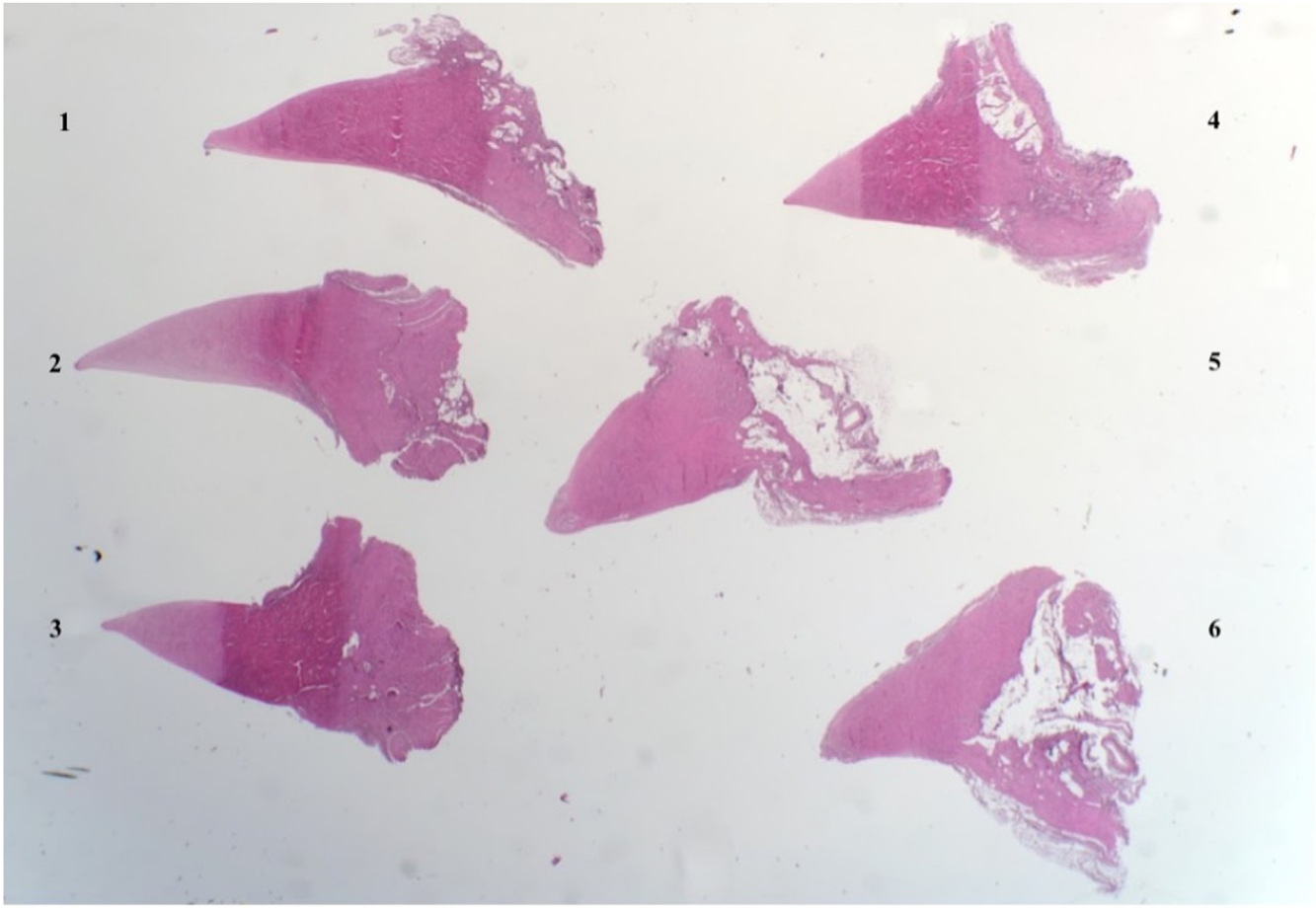


Fig 2. Medial meniscus with straight lines representing location of sections. Sections were labeled 1-6 on the slides; posterior root (1), posterior horn (2), body (3-4), anterior horn (5), and anterior root (6). Also shown are the (approximate) peripheral one-third and central two-thirds, marked by dotted black line.

camera. One image was centered on the peripheral one-third and one was centered in the central two-thirds of each of the 6 sections (2 images per section, 12 images per slide—each of these an area of 10×). FIJI imaging software was used to measure the cell count, cell density, and nuclear morphology (value of 1 representing perfect circle) of each image. FIJI imaging software is an open-source image processing package based on Image J, with provided tools to help facilitate scientific image analysis. In our study, FIJI was used to help visualize and analyze data obtained through light microscopy. Cell counts were evaluated automatically after thresholding each image for chondrocyte nuclei (Figs 4-6). The images of the meniscal sections pre-threshold and the threshold images were compared side by side to determine visually what was nuclei versus what was debris. If noted to be debris, the location was manually deleted from the threshold. To determine cellular density, the number of cells per visual field in a 10× image was divided by meniscal area within the 10× image. Nuclear morphology was obtained by automatic calculation of circularity of the selected nuclei in the Fiji system, with 1 representing a perfect circle. If there were 2 samples for one donor (left and right medial meniscus), these results were averaged to provide one value; there were 2 samples available for 10 donors.



Fige 3. Image demonstrating 6 sections of one meniscus placed on one slide. Posterior root (1), posterior horn (2), body (3-4), anterior horn (5), and anterior root (6).

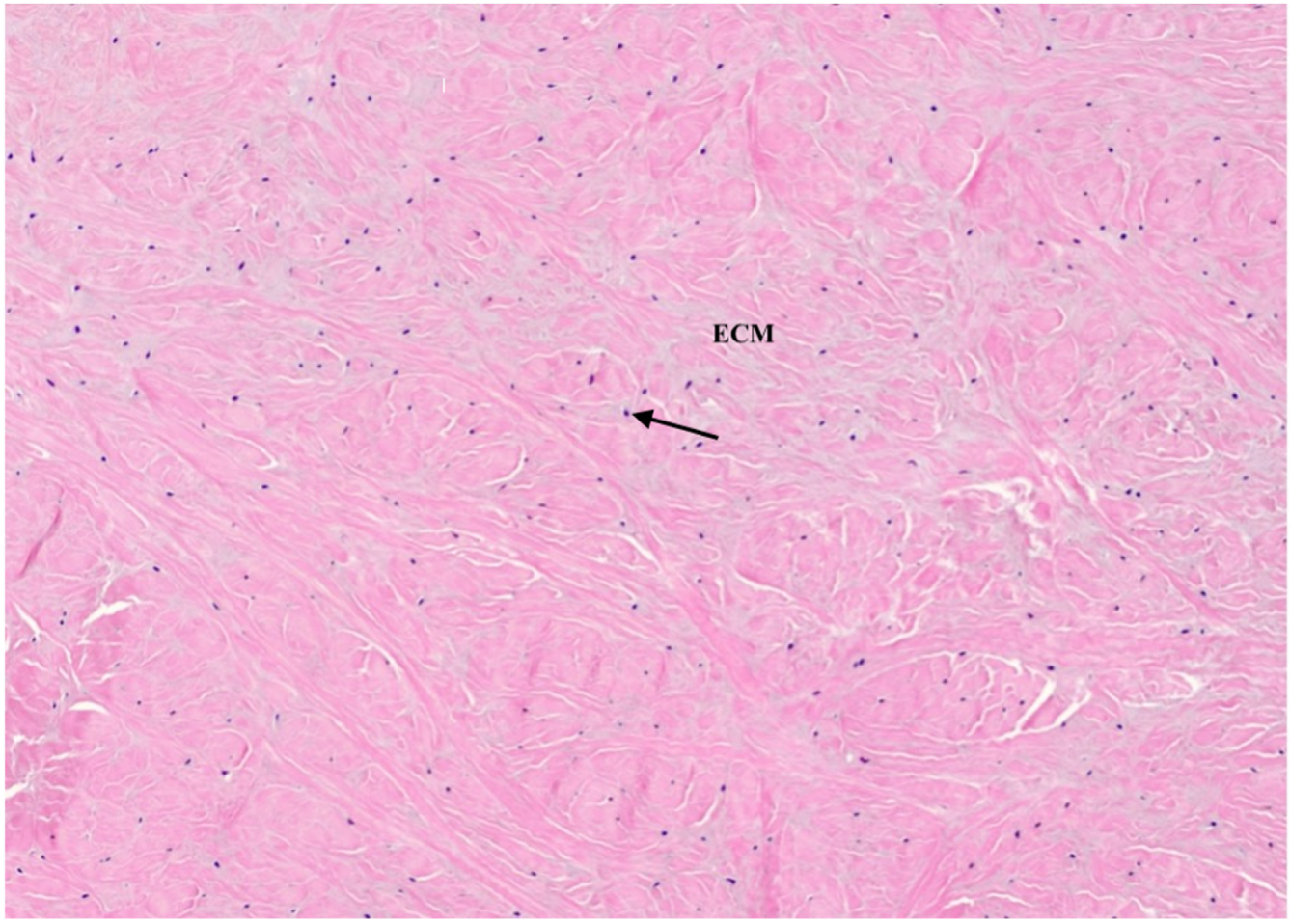


Fig 4. Photomicrographic image of meniscal section at 10× of 5-year-old male. Image with hematoxylin and eosin stain for evaluation of chondrocyte nuclei with the nuclei staining blue and the extracellular matrix and cytoplasm pink. Black arrow points to one chondrocyte nuclei stained blue. (ECM, extracellular matrix.)

Statistical Methods

To determine how cell count, nuclear morphology, and cell density relate to meniscal site (posterior root [1], posterior horn [2], body [3-4], anterior horn [5], and anterior root [6]), location (peripheral vs central), age, and sex, linear mixed models were fit for each response separately, with the 4 main effects (meniscal site, location, age, and sex) and all 2-way interactions as predictors, and individual as a random effect. Cell count and density were log transformed to achieve more equal variability and results back-transformed for reporting. Type II analysis of variance tests and pairwise tests with the Tukey correction were used to assess statistical significance, and least squares means and slopes calculated for terms of interest, with P values and standard errors reported as appropriate. $P < .05$ was considered statistically significant. Data analysis was completed in R, version 3.4.3 (2017-11-30; R Core Team [2017], R: A language and environment for statistical computing; R Foundation for Statistical Computing, Vienna, Austria).

Results

Cell Count

Cell count significance was found for meniscus section (1-6), location (central versus peripheral), the section/location interaction, and age ($P < .05$). There was no significance found based on sex. The peripheral menisci were found to be more cellular than the central menisci (Table 1).

The average cell count of the peripheral sections was found to decrease in the order of the anterior horn, anterior root, posterior horn, posterior root, and body (Fig 7). The average cell count of the central sections was found to decrease in the order of the anterior root, posterior root, anterior horn, posterior horn, and body (Fig 7).

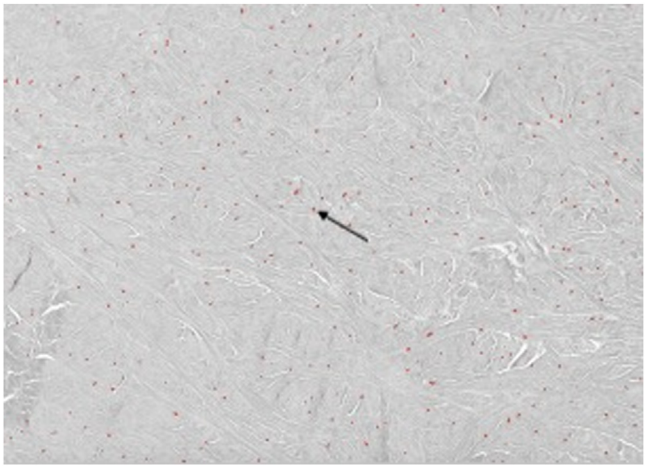


Fig 5. Threshold selection of isolated nuclei within the same meniscal (5-year-old male) section at 10× using Fiji software. Threshold selection was completed with Fiji imaging software to threshold each image for chondrocyte nuclei. This technique allows for the automatic selection of only the chondrocyte nuclei, shown in red in the above figure. Black arrow points to the same chondrocyte nuclei, now in red following threshold selection on imaging software.

The cell count was found to decrease by 14.1% per year of age in the horn (2 and 5), 13.6% per year of age in the body (3 and 4), and 13.4% per year of age in the root (1 and 6) of the meniscus. The cell count decreased by 14.1% per year of age in the central meniscus and 13.3% per year of age in the peripheral meniscus.

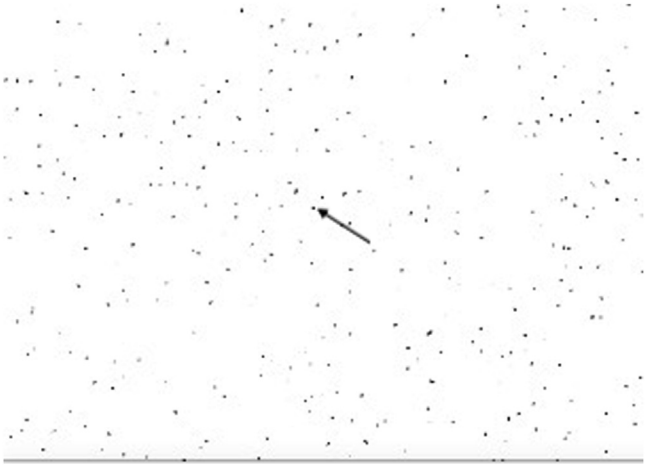


Fig 6. Final image following threshold from same meniscal section at 10× before software data collection—5-year-old male. This image depicts the isolated chondrocyte nuclei after threshold was completed, leaving only the nuclei to allow for automatic evaluation through the Fiji imaging software. Black arrow points to the same chondrocyte nuclei, now shown in black.

Table 1. Cell Count in Regard to Section (Cut); Posterior Root (1), Posterior Horn (2), Body (3-4), Anterior Horn (5), and Anterior Root (6)

Location	Section	Estimated Average Cell Count
Central	Posterior root	796
Central	Posterior horn	620
Central	Body	622
Central	Anterior horn	744
Central	Anterior root	1023
Peripheral	Posterior root	1124
Peripheral	Posterior horn	1181
Peripheral	Body	1081
Peripheral	Anterior horn	1239
Peripheral	Anterior root	1224

The overall cell count was found to decrease by 14% per year of age (Fig 8). The cell count in the periphery was found to decrease at a similar rate to the cell count in the central meniscus (Fig 8).

Cell Density

Cell density was significantly influenced by meniscus section, location, the section/location interaction, and age ($P < .001$), but not by sex. The meniscal cell density was approximately 2× greater peripherally than centrally (Fig 9). Cell density was relatively stable across sites in the peripheral meniscus but was somewhat greater centrally in the anterior and posterior roots than in the body and horns (Table 2).

In the central meniscus, the cell density was observed to have a 13.7% decrease per year of age in the roots (1 and 6), 14.3% decrease per year of age in the horns (2 and 5), and 13.9% decrease per year of age in the body. The cell density was found to decrease by 13.1% per year of age in the periphery and by 14.9% per year of age in the central meniscus. The differences between the rates of decrease per year of age were not found to be significant. The overall average cell density in all sites and locations in the menisci (sites and locations averaged) decreased by an average of 14% per year of age (Fig 10).

Nuclear Morphology

For nuclear morphology, there appeared to be an interaction between the section and location of the meniscus, with the cells in the peripheral meniscal body having a value closer to 1 (perfect circle) than those in the central meniscal body ($P < .01$) (Fig 11). There was no significance found for sex.

When analyzing section in relationship to age, there was a significant age/section interaction that appeared to be driven by a decrease in values for nuclear morphology (<1, less circular) across the sections in the 2 older donor samples (Fig 12). There was no significant overall age effect found across the section and location in ages <8 years old (Fig 12).

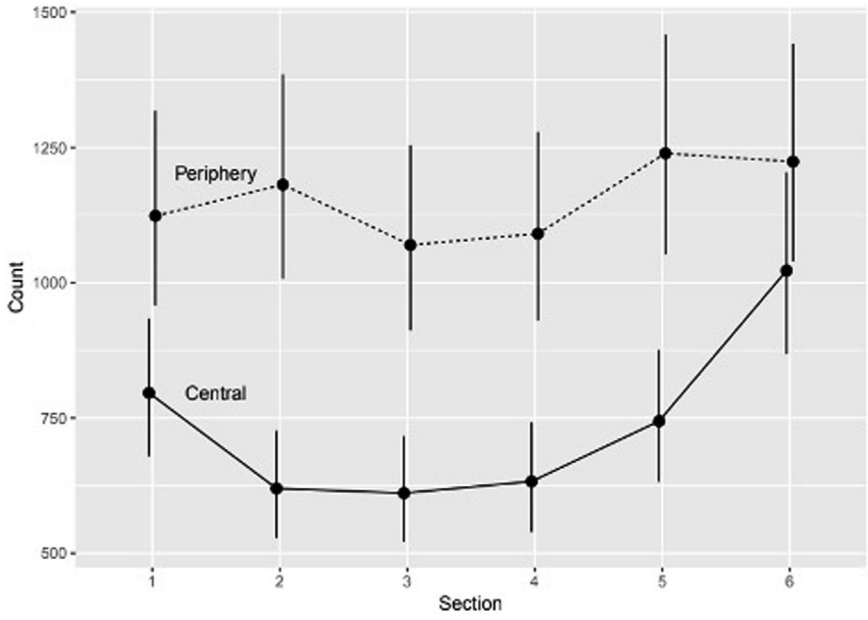


Fig 7. Cell count in regard to section and location; posterior root (1), posterior horn (2), body (3-4), anterior horn (5), and anterior root (6).

Discussion

The most important findings of this study are that there is a decrease in cell count as well as cell density of 14% per year of age, with a similar rate of decrease in peripheral versus central aspects of the meniscus. Overall, the peripheral meniscus was more cellular than the central meniscus at all ages. The nuclear morphology did appear to change in relation to age in both the peripheral and central locations, with the nuclear morphology decreasing in circularity with an increase in age in both locations, however, these results were not significant.

Previous studies have focused primarily on the prenatal development and structure of the human meniscus however, the number of studies on development of the meniscus in the skeletally immature knee is limited.[28-31] Clark and Ogden[18] investigated the developing menisci in pre- and postnatal cadaveric menisci in a study that included 28 postnatal menisci with an age range from 3 months to 14 years old. They

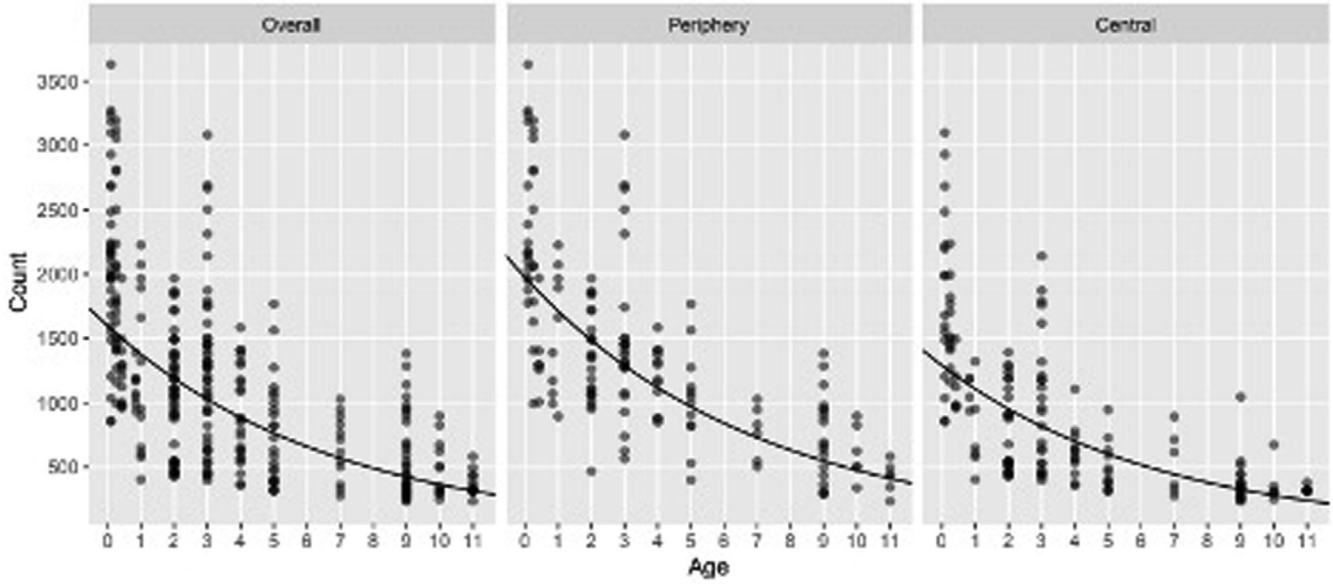


Fig 8. Rate of cell count decrease per year of age in order of overall, periphery and central locations. Each dot represents the cell count from one of the 6 meniscal regions from each meniscus sample. Darker areas of the dots represent multiple overlapping points.

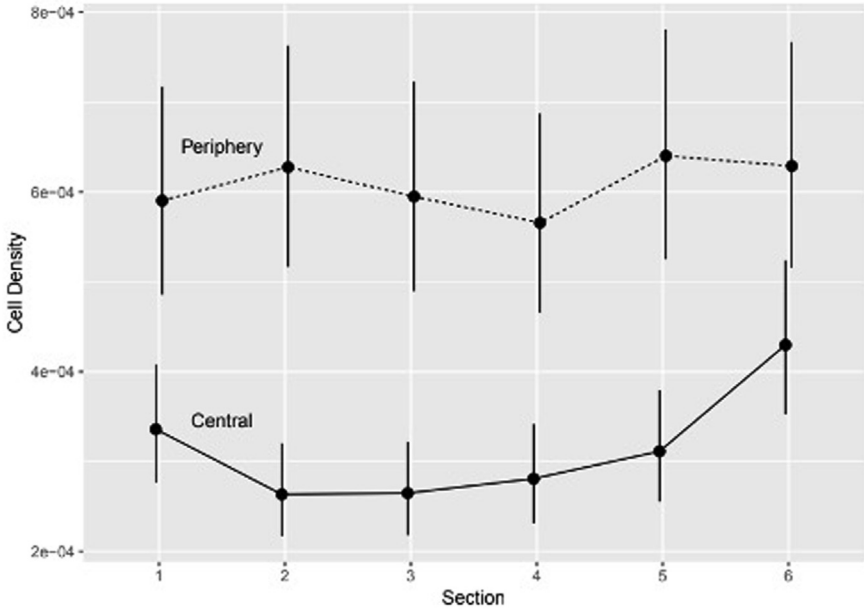


Fig 9. Cell density plotted by site (posterior root [1], posterior horn [2], body [3-4], anterior horn [5], and anterior root [6]) and location (central vs peripheral).

observed a decrease in cell density with age on histology. This study also noted a difference in density between peripheral and central locations, with the central one third of the meniscus having less cellular density than the peripheral meniscus based on microscopic observation. Similarly, our study found that cell density decreased with age and was greater in the periphery than centrally. However, the study analysis was done qualitatively by observation of histology slides. Our study expanded on this concept and used qualitative data to further assess the cellularity of the menisci and evaluated the nuclear morphology. In addition, our study evaluated these parameters based on section (roots, horns, body) in the meniscus. The cell count appeared to decrease at a similar rate in the roots, horns and body of the meniscus. Although the peripheral meniscus was more cellular, it did not have a faster rate of decrease in cell count than the central meniscus. The additional quantitative and location data may help us to better understand how the meniscus changes during skeletal maturity. It is clear that cellularity of the meniscus is changing directly from birth. Understanding the processes that cause this change and possibly intervening to slow down the change could result in improved longevity of the meniscus and joint preservation.

Table 2. Average Cell Density Based on Section: Posterior Root (1), Posterior Horn (2), Body (3/4), Anterior Horn (5), and Anterior Root (6)

Location	Section	Estimated Average Cell Density (Cell Count/Meniscus Area; cell/um^2)
Central	Posterior root	336
Central	Posterior horn	263
Central	Body	273
Central	Anterior horn	311
Central	Anterior root	430
Peripheral	Posterior root	590
Peripheral	Posterior horn	628
Peripheral	Body	581
Peripheral	Anterior horn	640
Peripheral	Anterior root	629

Field of view = 2 mm (10×/20).

Although literature discussing the change in cell count and density during skeletal growth in pediatric populations is sparse, multiple studies have investigated these topics in adult menisci.[2,6-10,12,13] Pauli et al.[12] looked at 107 healthy adult menisci with an age range of 23 to 92 years old, and similar to Clark and Ogden, the study investigated cellularity and density.[31] This study found an overall observed decrease in cell count and cell density of chondrocytes with increasing age in the adult population; the more degeneration present in the meniscus, the greater were the changes. Similar to Pauli et al.,[12] we found a decrease in cell count and density with increasing age, so it is likely that this is a continual process throughout growth. A mechanistic hypothesis for these changes relates to weight-bearing. We did not have enough information on weight-bearing in the donors to be able to assess pre- and postwalking; however, it may be that some of the changes within the meniscus are due to the changing loads on the meniscus during skeletal development.

There is one recent study that has looked at meniscal cellularity in skeletally immature human specimens.[32]

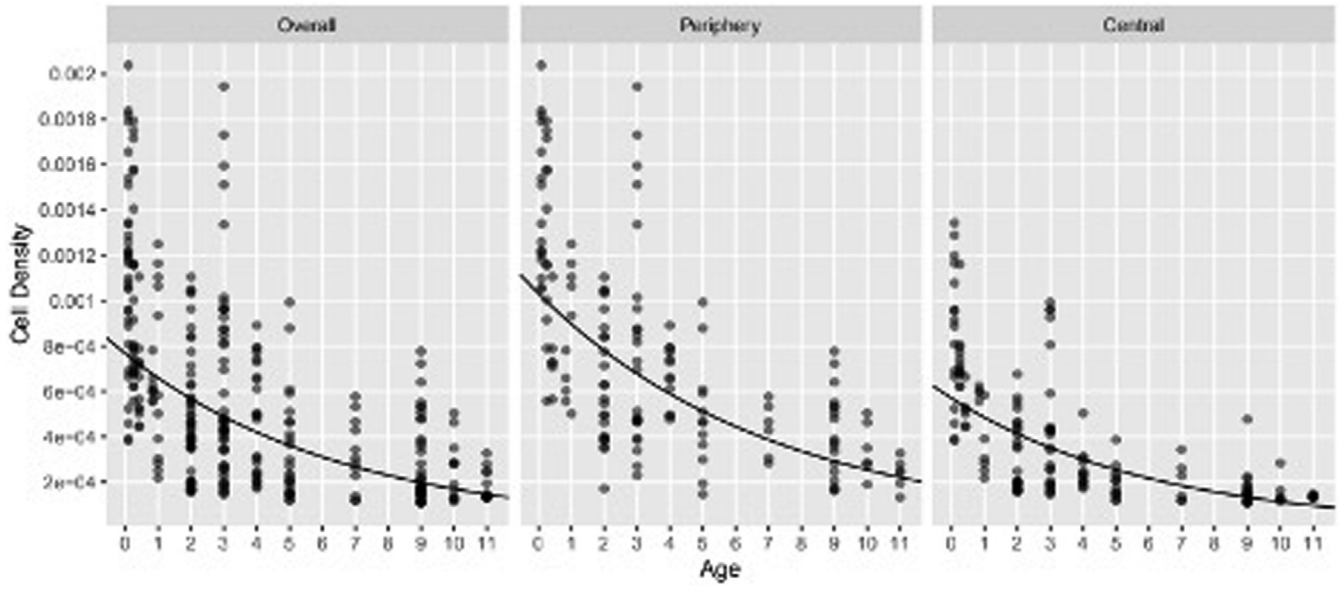


Fig 10. Cellular density versus age in years for meniscus overall and by central and peripheral location.

Among other things, the study looked at cell density and found that cell density decreased with age and that generally there was greater cell density in the roots, followed by anterior and posterior horns, followed by the meniscal body. Both of these findings are consistent with our study, although in the periphery of the meniscus we found greater cell density in the horns versus the root. Beyond the cell density data, our study adds to the previous study by providing information on meniscal nuclear morphology and cellular shape, which help inform about changes happening within the cellular activity of the meniscal cells over time.

Since skeletally immature human specimens are difficult to obtain, animal models have been used to look at the changing meniscus during skeletal development in greater detail. Meller et al.[33] investigated 10 maturing female sheep menisci at 1, 8, 18, 22, 28, and 40 weeks' postnatally. Their findings suggested a slight decrease in cell count with age, with this decrease becoming static after 18 weeks' postnatally. Bland and Ashhurst[34] also used an animal model, studying the menisci of rabbits with ages ranging from 20 days to 2 years old. This study similarly found a significant decrease in meniscal cell count and density in aging

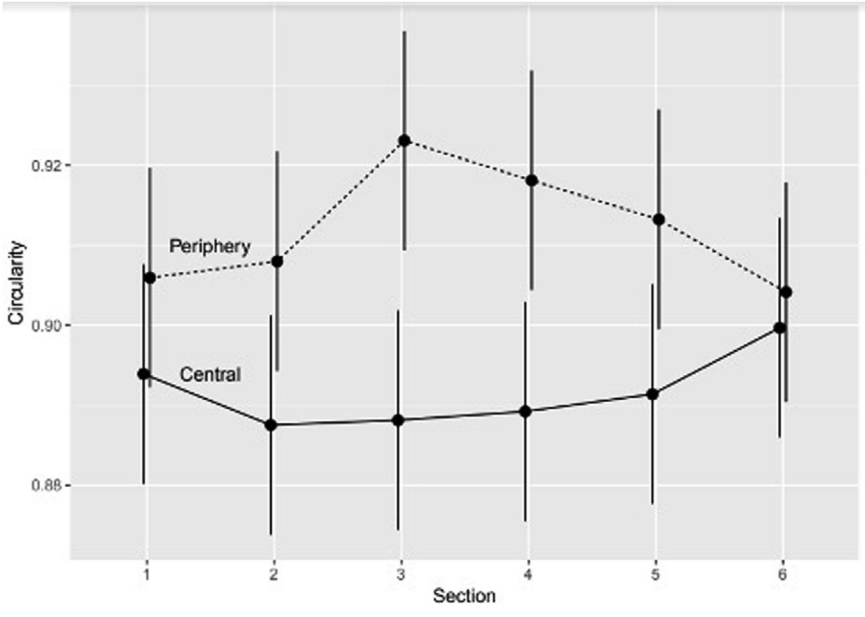


Fig 11. Circularity versus sections: posterior root (1), posterior horn (2), body (3-4), anterior horn (5), and anterior root (6). Value of 1 = perfect circle.

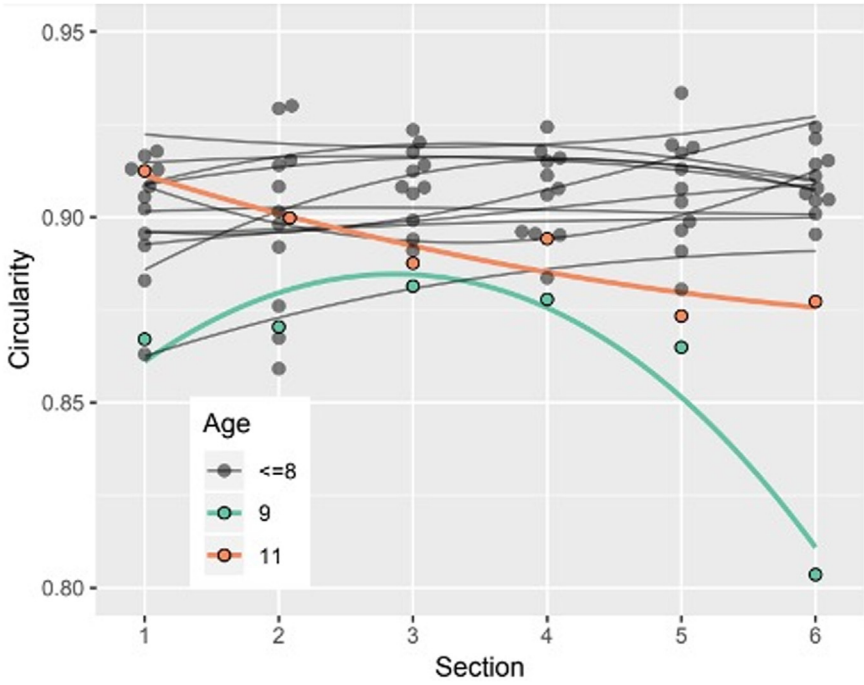


Fig 12. Meniscal nuclear morphology in relationship to age (in years) and section. This plot demonstrates the significant interaction found between age/section is driven by the 2 oldest subjects, both of whom have lower values for nuclear morphology as the section number increases.

rabbits. Although both studies are not human models, they do investigate the skeletally immature age group and demonstrate a change with age which suggests that the cellularity of the meniscus is changing directly from birth in both animals and humans.

This study focused on quantitative measures in the cells of the developing human meniscus. The data further define how the meniscus changes during skeletal maturity. Additional research focusing on the processes underlying these changes may lead to the creation of interventions to increase the longevity of the meniscus and preserve the joint.

Limitations

One limitation of this study is that we only used medial menisci in our study sample. Although the medial and lateral menisci are similar, they do have established differences in structural dimensions, and therefore the cellular composition of the medial and lateral menisci may be different as well.[32,35] Accordingly, this information can only be applied to the medial meniscus.

Conclusions

The results of this study reveal decreases in cell count, cell density, and circularity as age increases in cadaveric pediatric medial menisci.

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