Characterization of the Human Amniotic Membrane: Histological, Immunohistochemical and Ultrastructural Studies

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Abstract: Background and purpose: The human amnion has been applied clinically in the treatment of burn lesions, to cover surgical wounds, to avoid collusion and in ocular surface reconstitution. Amniotic membrane (AM) has many additional advantages, since such tissue is usually discarded, yet easily accessible and allows a very high recovery of cells. The aim of this work is to study the histological characterization of the human amnion membrane by using histological, immunohistochemical andultrastructural studies. **Material and Methods:** Amniotic membranes (AMs) were mechanically prepared from a placenta obtained with informed consent from healthy donor mother undergoing Cesarean section. The study and use of the AM was approved by the ethics committee of Al –Dawaadmi General Hospital. The AMs were taken and rinsed in phosphate buffered saline (PBS) and then fixed in 10% buffered formalin for general histology; 4% paraformaldehyde for immunohistochemistry and 5% gluteraldehyde fixative for Electron microscopy. **Results:** The AM is formed of epithelial layer, thick basement membrane (BM) and a vascular connective tissue (CT) layer. The epithelium is formed of one layer of cuboidal cells with rounded apex and vacuolated acidophilic cytoplasm and large rounded or oval vesicular nuclei with prominent nucleoli. The BM shows many processes and the CT stroma contains numerous collagen fibers run in different direction and mesenchymal cells. Few cells are positively immunostained with anti-smooth muscle actin; Anti-Vimintin; Anti-OCT3/4 and Anti- Nanog primary antibodies.Ultra structurally, the cell membrane exhibits numerous long microvilli and the lateral cell membrane shows complex interdigitation. The basal cell membrane is thrown in to many primary and secondary processes. Desmosomes are observed between the cells. The cytoplasm is filled with numerous bundles of tono filaments run in different directions and more concentrated around the nucleus. Supranuclear well developed Golgi, numerous free ribosomes, small strands of dilated rough endoplasmic reticulum (rER), small rounded vesicles and mitochondria are observed in the cytoplasm. Also, the cytoplasm contains lipid droplets. The basal processes are attached with the underlying CT by hemidesmosomes. **Conclusion:** This research has revealed information that the complicated structure of the epithelium of the human amnion has multiple specialized functions as it contains stem cells for organ transplantation and as an active secretory epithelium in addition to its function as covering epithelium**.**

[Abdul Rahman A. I. AL-Yahya and Madiha M.M. Makhlouf**. Characterization of the Human Amniotic Membrane: Histological, Immunohistochemical and Ultrastructural Studies.** *Life Sci J* 2013;10(4):3701-3710] (ISSN: 1097-8135). http://www.lifesciencesite.com**.** 498

Key words: Amniotic membrane - Immunohistochemistry - Ultrastructure– Histological.

1.Introdction

Human AM is the innermost fetal layer, lining the amniotic cavity and protecting the fetus during pregnancy. The outer layer, termed chorionic membrane, further separates the fetus from maternal tissues. The translucent, avascular, low immunogenic, anti-inflammatory, antiscarring, and wound healing properties of AM allow this material function beyond its role *in vivo* and assume a wide range of applications in regenerative medicine (Niknejad *et al.,* 2008 and Parolini *et al.,*2009). In fact, the clinical use of AM has a long history, with the first reports on its application in treatment of skin burns and wounds more than a century ago (Stern, 1913). These ground-breaking studies played a significant role in advancing the use of AM in surgery, especially in areas such as reconstruction of the corneal and conjuctival surfaces, treatment of open ulcers and traumatic wounds, and skin transplantation (Meller *et al*., 2011). In parallel, the shelf life of AM has been extended by irradiation, air-drying, lyophilization, cryo-preservation, and glycerol preservation techniques. These methods are expected to further expand the use of AM in ophthalmology to treat corneal, conjunctival and limbal lesions, burns, scars and defects as well as general surgery to reconstruct skin, genitourinary tract and other surfaces (Dobreva*et al.,*2010 and Kitagawa *et al.,*2011).

In particular, the expression of several cellular and molecular markers has confirmed the presence of stem cells in epithelial and mesenchymal stromal cultures. Subpopulations of both amniotic epithelial

(AE) cells and amniotic mesenchymal stromal (AMS) cells express pluripotency markers, including OCT4, SOX2, and NANOG (Zhao *et al.,*2005 and Tamagawa *et al.,* 2008).The main goal of this research is to study the characterization of the human AM using histological, immunohistochemical and ultrastructural methods.

2.Materialsand Methods

According to the policy approved by the local Ethical Committee (Al- Dawaadmi General Hospital), all tissue samples were obtained after informed consent. Term placentas from 6 healthy donor mothers obtained from caesarean sections were rapidly transferred to the laboratory, rinsed in phosphate buffer saline PBS containing penicillin and streptomycin (200 U/ml penicillin, 200 µg/ml streptomycin) and used immediately. The AM was separated from chorion through blunt dissection.The AMs were taken and rinsed in PBS and processed for light and electron microscopic examination.

Light microscopy:

Small pieces from the AMs were fixed in 10% buffered formalin. After fixation, specimens were dehydrated by using a series of ascending grades of alcohols (70, 90, 95 and absolute) then cleared in methyl benzoate or xylol and finally impregnated and embedded in paraffin. Serial sections were cut at 3-5 um and stained by Hx and E stain for general histological structure (Drury and Wallington; 1980).

Electron microscopy:

Small pieces from the AMs were fixed in 5% cold gluteraldehye for at least 24 hours then washed in 3-4 changes of cacodylate buffer (pH 7.2) for 20 minutes in each change and post fixed in cold osmium tetraoxide for 2 hours. The specimens were washed in four changes of cacodylate buffer for 20 minutes for each. Dehydration was done by using ascending grades of alcohol (30, 50, 70%) each for 2 hours and then 90%, 100% two changes 30 minute each. Embedding was done in Epon 812 using gelatin capsules for polymerization. The embedded samples were kept in incubator at 35^{o}C for one day, at 45^{o}C for another day and for three days at 60^{o}C (Gupta, 1983). Then semithin sections (0.5-1 microns) were prepared by using LKB ultramicrotome. The sections were stained with toludine blue, examined with light microscope and photographed. Ultrathin sections (50-80 nm) from selected areas of the trimmed blocks were made and collected on copper grides. The ultrathin sections were contrasted with uranyl acetate for 10 minutes, lead citrate for 5 minutes. Finally, the sections were examined and photographed by transmission electron microscopy (Jeol 100x) in Assiut University - Electron Microscopy Unit.

Immunohistochemistry:

Antibodies used in this studies were obtained from following sources; 1- Anti-Vimentin (mouse monoclonal, clone Vim V9; Dako, Glostrup, Denmark); 2- Anti-α-smooth muscle actin (clone 1A4); 3- anti-Oct-3/4, (H-134); and 4- anti-Nanog (H-155) (all polyclonal) Abs.

Staining protocol:

1-The specimens were immediately fixed in 4% paraformaldehyde for 48 hrs, then the specimens were put in 10% sucrose in 0.1M PBS for 6 hours and then put in 15% sucrose overnight at 4 degree and 30% sucrose for one day then frozen.

2- Frozen Sections (5-6 micron thick) were cut, mounted on poly L-lysine coated slides.

3 -Add hydrogen peroxide for 15 minutes to reduce non specific background staining due to endogenous peroxidase then wash 2 times in PBS for 5 minutes.

4- Apply Ultra V Block and incubate for 5 minutes at room temperature to block non specific background staining.

5- Wash the slides in PBS and stain with alkaline phosphatase for 10 min. or until the color of the slides is changed to red,then wash the slides in PBS.

6- Add the primary antibody (Anti-Nanog, Anti-smooth muscle actin, Anti – vimentin and anti-Oct-3/4), for one hour at room temperature then wash 4 times in PBS.

7- Apply Biotinylated mouse Anti-Polyvalent (secondary antibody) and incubate for 30 minutes at room temperature then wash 4 times in PBS.

8- Apply Streptavidin Peroxidase and incubate for 10 minutes at room temperature then rinse in PBS and stained with nuclear stain Hoechst 33258 then the sections were mounted and examine by Fluoresence microscope (Cerson, 1990 and De Cock *et al*., 1997).

Negative control slides were done by omitting the primary antibody to ensure that the immunostaining is true.

3.Results and Discussion:

Amniotic epithelial(AE) cells are anatomically and histologically specialized fetal epithelial cells that normally exist less than 10 months in nature. As shown here, the AE cells derived from term placenta seem to remain somewhat “plastic” in their differentiation options and maintain the capability to differentiate and contribute to cells from all three germ layers. The focus of our experiments was concentrated on the character of AE cells and identification of stem cells using different markers.

Using Hx&E stained sections reveals that, the AM is formed of epithelial layer, thick BM and a vascular connective tissue layer. The epithelium is formed of one layer of cubical cells with rounded

apex, vacuolated acidophilic cytoplasm and rounded or oval nuclei (Fig.1). The BM shows many processes and the c.t. stroma contains collagen fibers and mesenchymal cells(Fig.1). Examination of semithin sections stained with Toluidine blue shows that, the epithelium of the AM is formed of one layer of high cubical cells with rounded apex and vacuolated cytoplasm. The nuclei are vesicular rounded or oval with prominent nucleoli and irregular outline. They are present in the apical part of the cells (Figs. 2&3). The basal cell membrane shows many processes (Fig.3). The cells are rest on athick homogenous BM (Fig.3). Few cells appear degenerated and detached from the epithelium (Fig. 3). The CT stroma contains numerous mesenchymal cells of varying shapes and sizes and bundles of collagen fibers run in different directions (Figs.2&3).

The present results are in agreement with the results of Niknejad *et al*. (2008) who found that the AM consists of an epithelial monolayer, a thick BM, and an avascularstroma.The AM contains no blood vessels or nerves; instead, the nutrients it requires are supplied directly by diffusion out of the amniotic fluid and/or from the underlining *decidua*.

The BM is one of the thickest membranes found in all human tissue. The support provided to the foetus by the BM throughout gestation stands testimony to the structural integrity of this remarkable membrane. The compact layer of stromal matrix adjacent to the BM forms the main fibrous skeleton of the AM. The collagens of the compact layer are secreted by mesenchymal cells situated in the fibroblast layer. Interstitial collagens (types I and III) predominate and form parallel bundles that maintain the mechanical integrity of AM. Collagens types V and VI formfilamentous connections between interstitial collagens and the epithelial BM (Parry and Strauss, 1998).

The AM, lacking any vascular tissue, forms most of the inner layer of the fetal membrane (Ilancheran; *et al*.;2007 and Pappa and Anagnou, 2009) and is composed of 3 layers: (i) an epithelial monolayer consisting of epithelial cells, (ii) an acellular intermediate basement layer, and (iii) an outer mesenchymal cell layer, rich in mesenchymal stem cells and placed in close proximity to the chorion (Ilancheran *et al*.; 2007 and Pappa and Anagnou, 2009). The AM was used in clinic for many decades for wound healing in burns, promoting epithelium formation and protecting against infection (Bose,1979 and Hao, *et al.,*2000). The use of AM has been evaluated as a wound dressing material for surgical defects of the oral mucosa (Arai,*et al.,* in press), ocular surface reconstruction (Kim and Tseng,1995 and Hao *et al.,*2000), corneal perforations (Kitagawa *et al.,*2009 and Kitagawa *et al.,*2011)and bladder augmentation (Iijim *et al.,*2007).

Regarding the immunohistochemistry,using immunofluorescent microscopy the AE and mesenchymal cells show positive expression of several stem cell(SC) marker molecules. Figures (4-7) shows the representative images of immunostaining with anti-smooth muscle actin, anti-vimintin, anti-OCT ¾ and Anti-Nanog. Stem cell surface markers are present on AE cells. AE cells express Monoclonal mouse Antihuman smooth muscle actin, Vimentin, OCT3/4 and Nanog.In particular, the expression of several cellular and molecular markers has confirmed the presence of stem cells in epithelial and mesenchymal stromal cells.

Amniotic membrane stem cells (AMSCs) include two types, the amniotic epithelial cells (AECs) and the AMmesenchymal stem cells (AM-MSCs) (Pappa and Anagnou, 2009, and Cai *et al*., 2010). Both cell types are originated during the pregastrulation stages of the developing embryo, before the delineation of the three primary germ layers and are mostly of epithelial nature (Parolini,*et al., 2008*). A variety of protocols have been established for AECs and AM-MSCs isolation, primarily based on the mechanical separation of the AM from the chorionic membrane and the subsequent enzymatic digestion (Miki *et al.,*2010 and Marongiu *et al.,*2010). AM-MSCs exhibited plastic adherence and fibroblastoid morphology, while AECs displayed a cobblestone epithelial phenotype. AM-MSCs shared similar phenotypic characteristics with the ones derived from adult sources. More interestingly, AM-MSCs, exhibited a higher proliferation rate compared to MSCs derived from adult sources and a multilineage differentiation potential into cells derived from the three germ layers (Manuelpilla *et al.,*2011).Vimentin is a cytoskeleton protein from mesenchymalcells. Interestingly enough, immunohistochemical data demonstrates that HAE cells in situ express two types of intermediate filaments within the same cell, cytokeratin and vimentin. This reforces previously published approaches where HAE cells, despite their clearly ectodermal-epidermal origin not only expressed general epithelial markers such as cytokeratins (Beham *et al.,* 1988) or epidermal markers such as CA 125 (Nanbu et al., 1989b), but also expressed a marker for mesenchymally derived cells, vimentin (Wolf *et al.,* 1991; Sakuragawa*et al.,* 1996, and Uchida*et al.,* 2000c).

Intermediate filaments play an important role in the differentiation and proliferation ofcells. During differentiation, transdifferentiation and neoplastic formation, dramatic changes occur in the intermediate filament protein expression and organization(Bouwens *et al*., 1997). This expression has been specifically

studied in thepancreatic system. Vimentin positive cells have been observed in epithelial ducts during embryogenesis (Rosenberg, 1995), in the differentiation from adult toprogenitor ß cells (Zulewski *et al*., 2001), and during ductal cell proliferation during carcinogenesis (Petersen *et al*., 2003). Consequently, vimentin in pancreatic duct cells can be considered a useful marker for pancreatic precursor cells (Ko *et al*., 2004). Taking into account that vimentin is expressed in cultured HAE cells, their ability to differentiate into pancreatic precursor cells could be especulated. This hypothesis is enforced by the Wei *et al*.(2003) study, where HAE cells were differentiated to secrete insulin.

Oct-4 and Nanog are well-accepted molecules to define SCs (Boyer *et al*., 2005 and Loh, *et al*.; 2006). Our cells clearly expressed both molecules.

Ultrastructurally, the cells of the human AM are high cubical cells. The apical cell membrane exhibits numerous long microvilli and the lateral cell membrane shows complex interdigitation. The basal cell membrane is thrown into many primary and secondary processes(Figs 8&9). Desmosomes are observed between the cells(Figs. 16&17). The cytoplasm is filled with numerous bundles of tonofilaments run in different directions and more concentrated around the nucleus (Fig. 9). Supranuclear well developed Golgi bodies, numerous free ribosomes, small strands of dilated rER, small rounded vesicles andmitochondria are observed in the cytoplasm (Figs 10&11). Also, the cytoplasm contains lipid droplets and secretory vesicles (Figs. 8&9&12B). The basal processes are attached with the underlying connective tissue by hemidesmosomes (Fig. 17). The nucleus appear large euchromatic with irregular nuclear membrane and peripheral clumps of heterochromatin (Figs.9&12A). The process of degeneration is observed clearly: at first, the cell shows shrunken irregular nucleus with wide intercellular spaces (Fig. 13), after that the the nucleus appeared bilobed with coarse clumps of marginated heterochromatin; wide intercellular space and shrunken basal processes (Fig. 14). Then the cell appear shrunken with pyknotic nucleus and detached from the epithelium (Fig.15).

The BM is thick and formed mainly of collagen fibers. The CT stroma contains collagen fibers and undifferentiated mesenchymal cells (Figs.18-20). The present study is in agreement with that described by Jingwei Hu *et al*. (2009) who stated that the transmission electron microscopy of hAMCs reveals ahybrid epithelial–mesenchymal ultrastructural phenotype: epithelial characteristics include non-intestinal-type surface microvilli, intracytoplasmiclumina lined with microvilli, and intercellular junctions. Mesenchymal features include rough endoplasmic reticulum profiles, lipid droplets, and well-developed foci of contractile filaments with dense bodies (Pasquinelli *et. al*.; 2007). These features are consistent with the view that hAMCs have pluripotent potential. Transmission electron microscopy shows that hAECs are cuboidal, with apical microvilli, and that their lateral cell borders are convoluted with frequent desmosomes and no obvious tight junctions. The basal epithelial cell surfaces are highly convoluted with frequent hemidesmosomes at the distal termini of cell processes, and wavy filament bundles are seen in the adjacentcytoplasm (Aplin *et al*.;1985).

It is necessary to remark that the AE is composed of unique amnioticepithelial cells exhibiting quite characteristic morphological features. They have a relatively large numbers of intracytoplasmic organelles, microvilli on the apical surface, abundant cytoplasmic processes to the lateral and basal sides, and loose intercellular connections (Matsubara and Sato, 2000).

The process of degeneration in our results are in agreement with the results of Runic *et al*.(1998) who found apoptosis in the nuclei of the AM using TUNEL/immunohistochemical method and Electron microscopy. The amnion epithelial cell layers consistent with apoptosis, including condensation of nuclear chromatin along the periphery of the nucleus and shrinkage of cellular cytoplasm. Peripheral condensation of chromatin, a hallmark of apoptosis, was apparent within highly condensed nuclei (Lazebnik *et al.,* 1993). Morphological characteristics of apoptosis include condensation of chromatin along the periphery of nuclei, nuclear shrinkage, and subsequent loss of membrane integrity and formation of apoptotic bodies (Lazebnik *et al.,* 1993).

Data obtained by electron microscopy in thepresent study are consistent with changes noted during apoptosis, including condensation of chromatin along the periphery of the nucleus and, ultimately, shrinkage of the nucleus and the cell itself. In conclusion, this study has revealed information that the complicated structure of the epithelium of the human amnion has multiple specialized functions as it contains stem cells for organ transplantation and as an active secretory epithelium in addition to its function as covering epithelium**.**

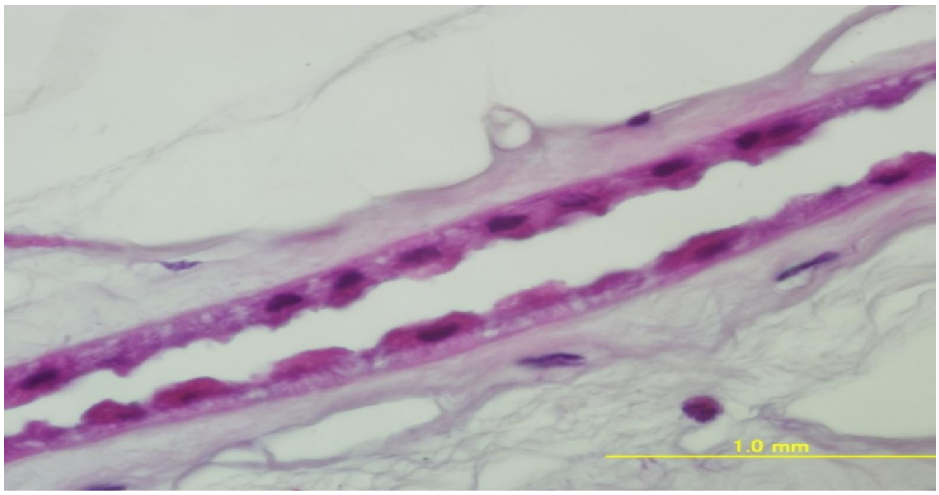

Fig:1: A section in the amnion membrane stained with Hx&E showing the lining epithelium and underlying connective tissue.(Hx&E X1000).

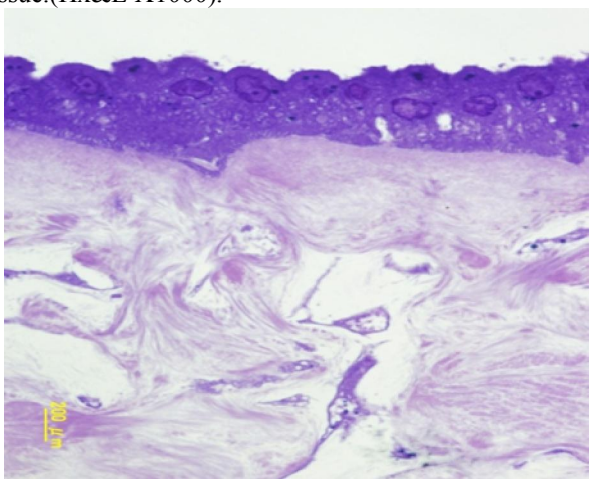

Fig.2: A semi thin section in the amnion membrane showing the lining epithelium, which is formed of high cuboidal cells with rounded apex and numerous basal processes. (Toluidine blue X1000).

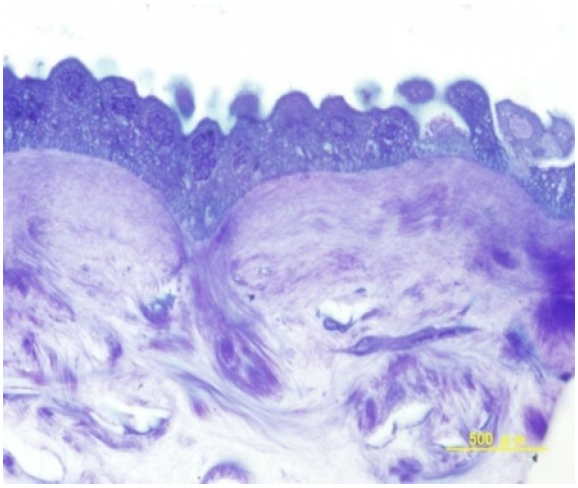

Fig.3: A semi thin section in the amnion membrane shov degenerated cells pinched off from the lining epithelium blue X1000).

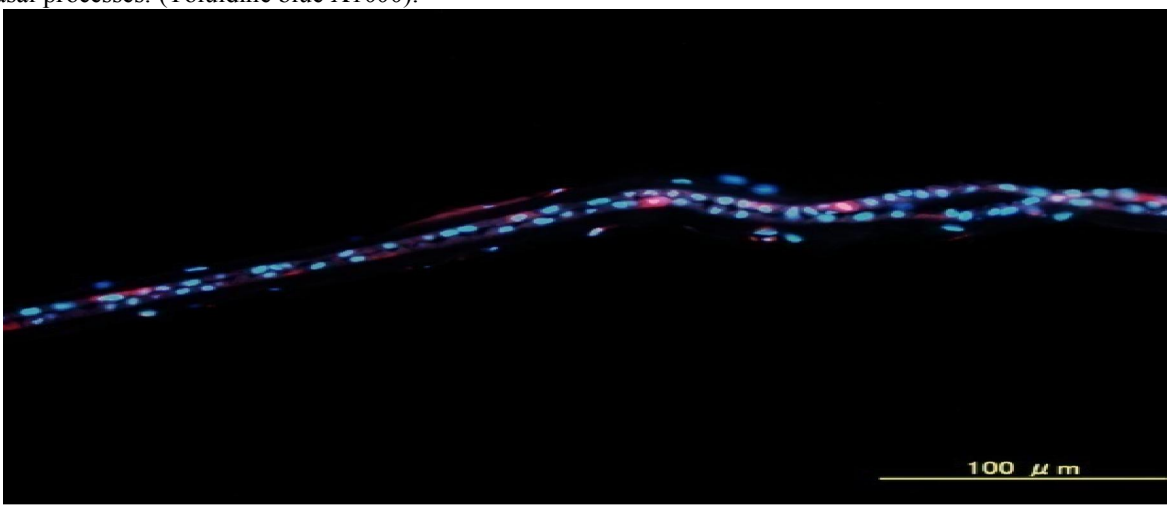

Fig. 4: Amnion membrane immune stained with ALP. And anti-smooth muscle actin primary antibody showing; few positive stem cells. (X200).

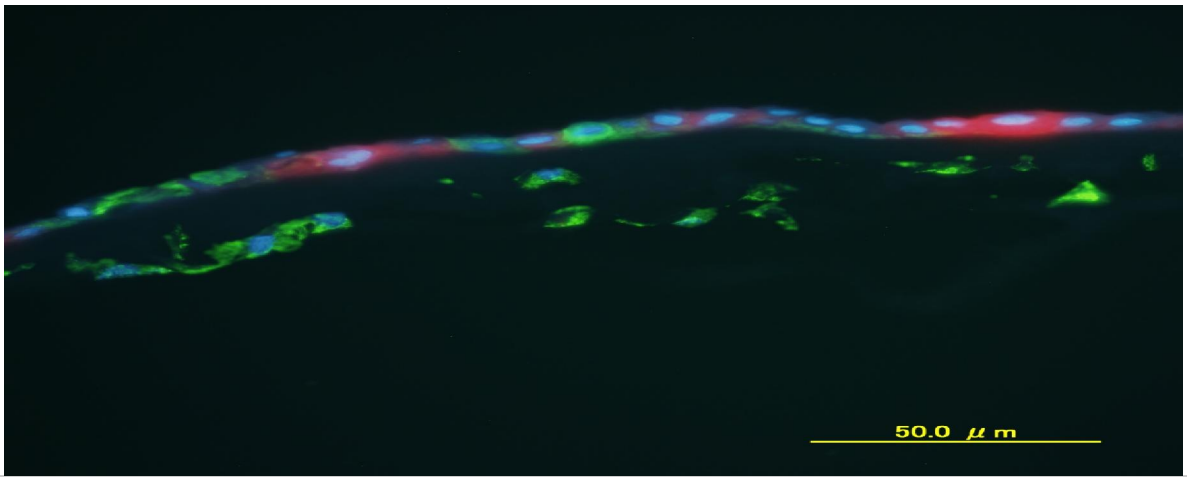

Fig.5: Amnion membrane immune stained with ALP. And Anti- vimentin primary antibody showing; the positive stem cells in the epithelial lining and in the underlying mesenchymal connective tissue cells. (X400)

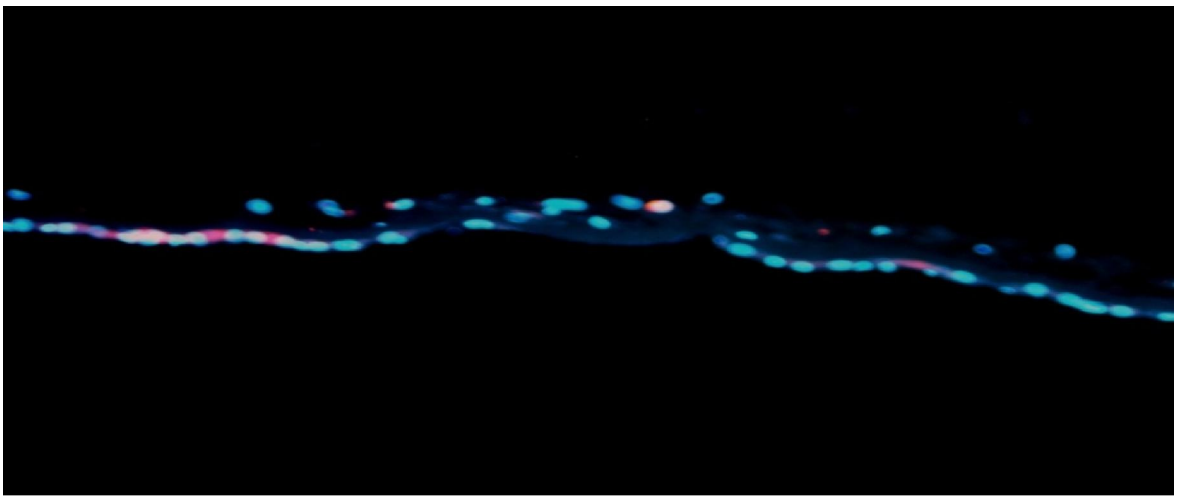

Fig.6: Amnion membrane immune stained with ALP and Anti-OCT3/4. Primary antibody Showing; the few positive stem cells. (X400).

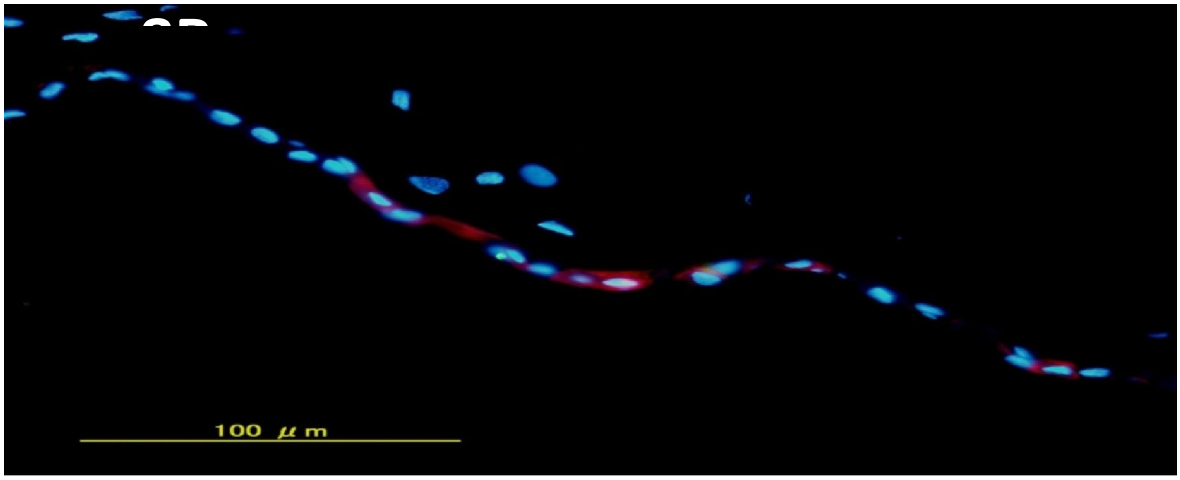

Fig.7: Amnion membrane immune stained with ALP. And Anti-Nanog primary antibody showing; the positive stem cells. (X400).

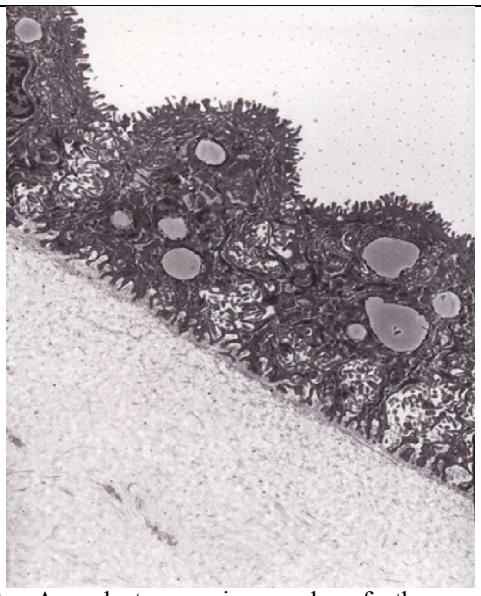

Fig.8: An electron micrograph of the amnion membrane showing; the epithelial lining and underlying connective tissue. (X3000).

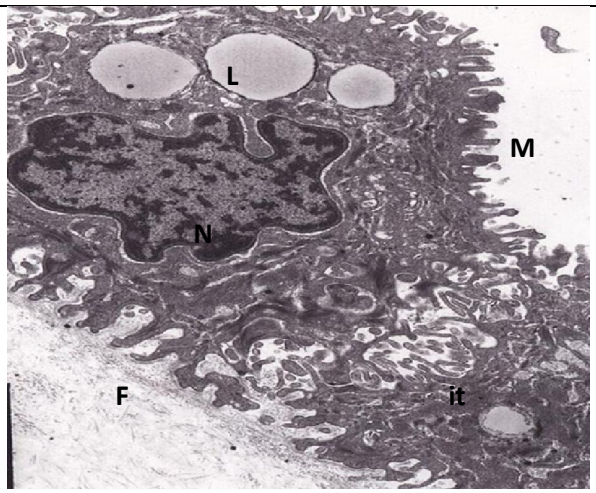

Fig.9: A magnified part of the previous section showing, apical microvilli (m), basal processes (P) and complex lateral interdigitations (IT). The cytoplasm is studied with numerous tonofilaments (T) closely related to the nucleus. Multiple lipid droplets (L). Note the large irregular nucleus (N) with coarse clumps of peripheral heterochromatin.(x 6000).

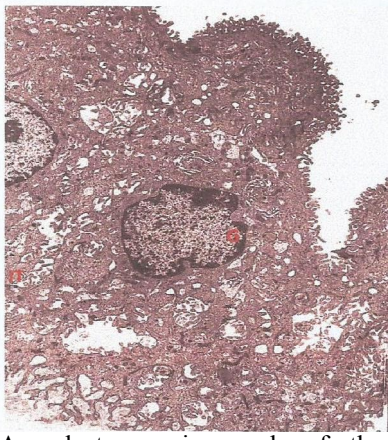

Fig.10: An electron micrograph of the amnion epithelium showing; Golgi bodies (G) and complicated interdigitations. Note the apical part of the cell is going to be separated. (X3000).

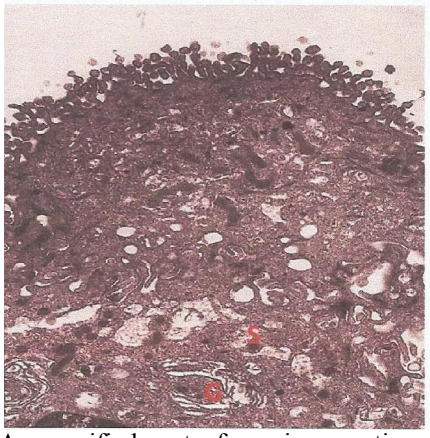

Fig.11: A magnified part of previous section showing; well-developed Golgi bodies (G) and multiple spaces (S) at the line of separation between the apical part and the rest of the cell.(x 7,300).

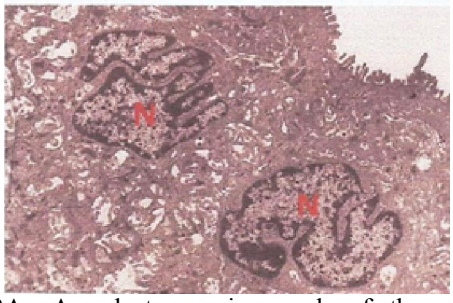

Fig.12A: An electron micrograph of the amnion epithelium showing;highly intended nuclei(N) at several situations.(X3000)

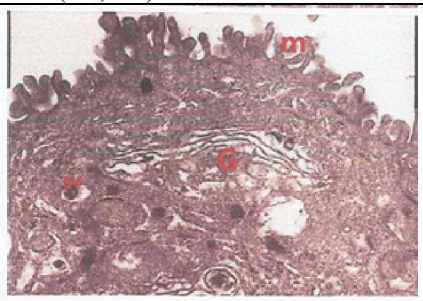

Fig.12B: A magnified part of previous section showing; numerous apical microvilli (m). Note the well-developed Golgi complexes (G) and secretory vesicles (sv) in the cytoplasm. (X10000).

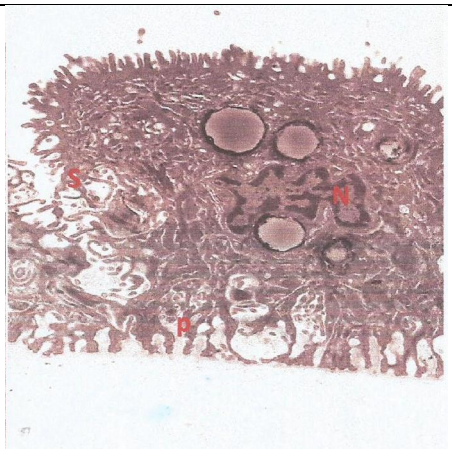

Fig.13: An electron micrograph of the amnion epithelium showing; the shrunken irregular nucleus (N) with wide intercellular spaces (S) as well as irregular basal processes (P). (X5000).

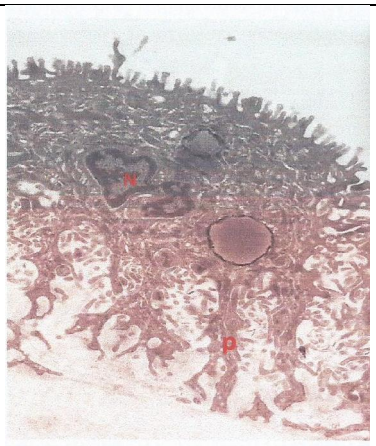

Fig.14: An electron micrograph of the amnion epithelium showing; the bilobed nucleus (N) with coarse clumps of marginated heterochromatin. note the increase in the width of intercellular spaces as well as basal processes (P). (X6000).

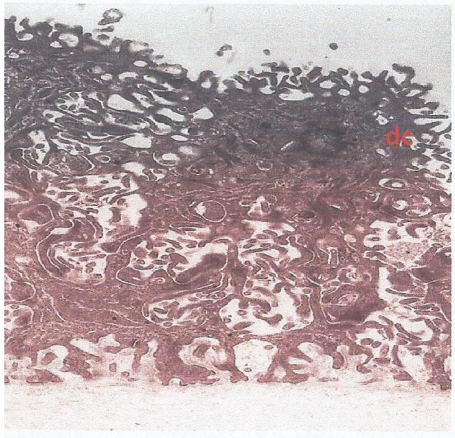

Fig.15: An electron micrograph of the amnion epithelium showing; degenerated cell (dc) with pyknotic nucleus. This cell is going to detached from the adjacent cells. (X6000).

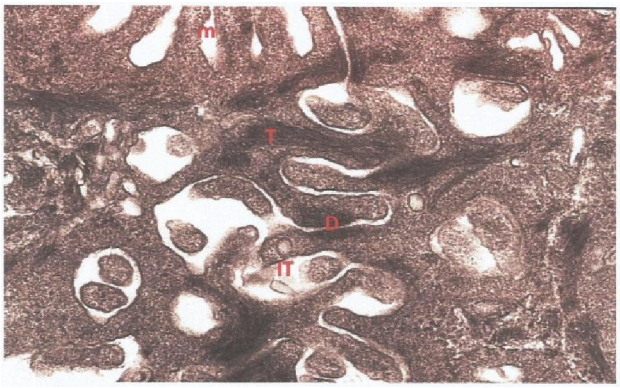

Fig.16: An electron micrograph of the apical part of the amnion epithelium showing; numerous apical microvilli (m), a huge number of tonofilaments (T) as well as lateral interdigitation (IT).
Note: the presence of well developed desmosome between the cells. (x20,000).

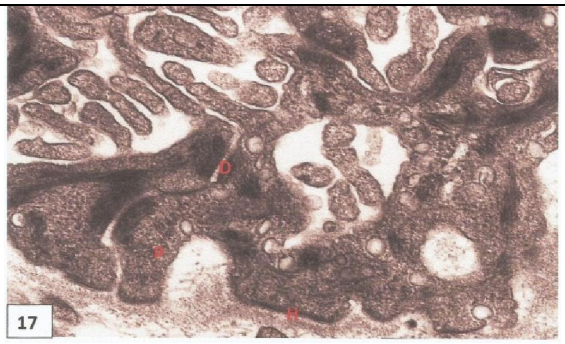

Fig.17: An electron micrograph of the basal part of the amnion epithelium showing; well-developed desmosomes (D), hemidesmosomes (H) and basal processes (P). (x20,000).

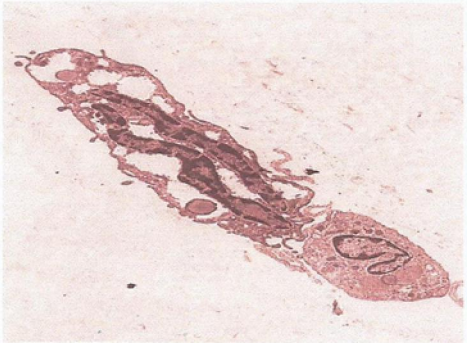

Fig.18: An electron micrograph of the amniotic connective tissue showing; undifferentiated mesenchymalcells. (x 3,700).

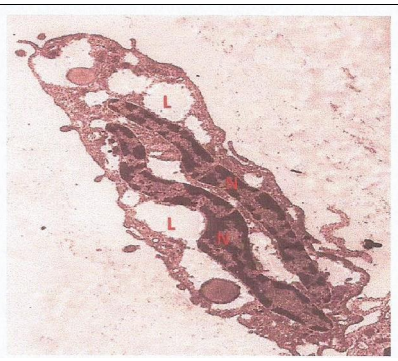

Fig.19: A magnified undifferentiated mesenchymal cell appear flattened with irregular flattened hetrochomatic nuclei and lipid rich scanty cytoplasm.(x6,000).

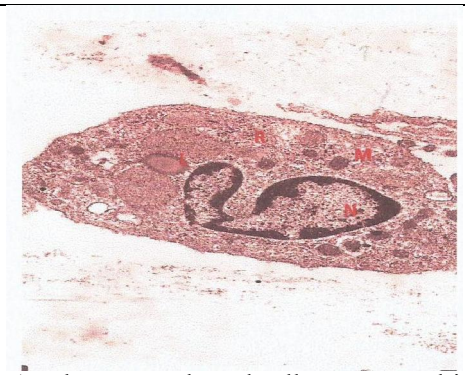

Fig.20: Another mesenchymal cell appears oval in shape with indented hetrochomatic nucleus (N), lipid droplets (L), multiple mitochondria (M) and numerous free ribosomes (R) are present in the cytoplasm. (x10,000).

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