Co-culturing improves induction of human amniotic epithelial cells into corneal epithelial-like cells *in vitro*

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Co-culture system has the feasibility of trans differentiating multipotential cells into specific cells, which plays an important role in tissue engineering. Corneal alkali burn is a common type of ocular injury, which often results in extensive damage and permanent visual impairment. Recently, human amniotic epithelial cells (hAECs), a type of multipotent cells originally isolated from amnion tissues, have become a promising source for the treatment of corneal chemical burn. Accumulating evidence has indicated that hAECs possess multi-differentiational properties for tissue repair and regeneration. Here, we present evidence that hAECs possess immunophenotypic features of corneal epithelial cells and ultrastructure changes, indicating that these cells have the potential to differentiate into corneal epithelial-like cells. Our findings therefore suggest that hAECs could be a promising substitute for corneal epithelial cells which are widely damaged by alkali burns.

Keywords: Corneal epithelial-like cells, co-culture, corneal alkali burn, human amniotic epithelial cells.

ALKALI burn of ocular surface tissue is a bilateral clinical problem and often leads to a loss of vision resulting from ulceration, scarring and neovascularization during healing[1]. Although major advances have been made in the treatment of corneal epithelial defect resulting from alkali burn, including the use of corneal allotransplantation and stem cell transplantation, the failure of corneal transplantations performed after corneal chemical injuries due to immunologic rejection is still an intractable problem[2,3]. Meanwhile, the availability of suitable donor cornea is limited. Stem cell transplantation is deemed to be an effective treatment but possesses ethical controversy and relatively limitative obtainment; these limitations seriously offset its use.

Many researchers have shown that amniotic membrane has been widely applied to oculopathy. For example, amniotic membrane transplantation for treating corneal alkali burn and corneal ulcer. Human amniotic epithelial cells (hAECs) isolated from the human amniotic membranes have considerable advantages over other sources of stem cells such as embryonic or bone marrow-derived cells. They are abundant, multipotent, with low immunogenicity, lack of tumorigenicity and uncontroversial to their collection because the amniotic membrane is discarded after delivery[4,5]. It has been shown that hAECs have the potential to differentiate into ectoderm cells (neural cells), mesodermal cells (cardiomyocytes) and endodermal cells (hepatocytes and pancreatic cells) *in vitro*[6–9]. Meanwhile, hAECs can secrete various cytokines that perform multiple biological functions, including anti-inflammatory, anti-angiogenesis, neurotrophy on organ or tissue injury. Based on these characteristics, hAECs are considered to be as useful biological materials and also a potentially superior cell source to complement or replace damaged corneal cells.

The purpose of this study was to identify hAECs cultured by tissue cultivation and investigate the cellular capacity of differentiation into corneal epithelial-like cells through morphological and biochemical analyses. Here we report the ability to induce hAECs into corneal epithelial-like cells by co-culture with corneal epithelial cells (CECs) *in vitro*, providing a valuable tool for a potential cellular therapy for corneal alkali burn.

In accordance with the Declaration of Helsinki, an informed consent was signed and hAECs were obtained from the placentas of healthy women (the placentas would usually be discarded after delivery)[10]. The research procedure was approved by the Ethics Committee of the Harbin Medical University, China. All infectious pathogen-positive deliveries involving HBV, HCV and HIV were excluded. The amnion manually stripped from the chorion was washed in PBS containing 100 U/ml penicillin and 100 μg/ml streptomycin (Hyclone, Waltham, MA, USA), and cut into small pieces. The amniotic tissues were placed in six-well plates (NEST, Peking, China) and incubated with three drops of foetal bovine serum (FBS, Hyclone) for 2 h at 37°C in a humidified atmosphere containing 5% CO_2. Next, compound culture medium was added composed of Dulbecco's modified Eagle's Minimum Essential Medium (DMEM, Hyclone), 10% FBS, 1% (v/v) penicillin–streptomycin and 10 ng/ml epidermal growth factor (EGF, Solarbio, Peking, China) into six-well plates and the tissues were kept in an incubator. In accordance with the routine culture, the medium was changed every other day.

Cells were passaged when reaching 80% confluence. On being after digested with 0.25% trypsin (Hyclone) for 5 min at 37°C, the aforementioned compound culture medium was added to terminate dissociation and inactivate trypzean (Hyclone). HAECs were collected by centrifugation and resuspended in culture solution. The cells were cultured in the culture medium according to a previously reported protocol[11]. Coverslips were placed at the bottom of the plates and hAECs cultured in medium from the second passage were seeded on cover glasses at a density of 5×10^4 cells per sq. cm. The cells were cultured for 24 h, and washed three times with phosphate-buffered saline (PBS) and fixed with 10% formaldehyde,

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and then stained with haematoxylin-eosin (HE) for microscopic observation. To demonstrate the results, photomicrographs were obtained. Morphological characteristics were observed under an inverted microscope (Olympus, Japan).

For isolation and culture of CECs, 4–6-weeks-old white New Zealand rabbits were housed in a standard animal laboratory. All of the experimental protocols in this study were approved by the Ethics Committee of Harbin Medical University; all work was conducted in accordance with the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rabbits were executed by air embolism after the delivered overdose of chloral hydrate. The corneas were extracted and then cut into 2×2 cm^2 pieces with surgical scissors. They were cleaned with PBS containing antibiotics at 37°C. According to the above-mentioned hAEC explant cultures, the cell medium was exchanged every other day. The growth of CECs was observed daily using an invert microscope. Images were taken with an digital camera (Canon, Japan).

Immunohistochemistry analysis was performed to identify hAECs; the second passage hAECs were seeded on cover glasses (ShiTai, Jiangsu, China) at a density of 5×10^4 cells per cm^2 and cultured in standard medium. After 1 week the cultured cells were washed three times with PBS and fixed in 4% paraformaldehyde for 15 min, and then again washed three times in PBS (5 min each). The cells were permeabilized with 0.1% Triton-X-100 (Sigma, USA) for 15 min and washed three times in PBS (5 min each), and subsequently dipped in 3% hydrogen peroxide solution for 10 min. Cells were blocked with 4% bovine serum albumin (BSA, Sigma) for 30 min at room temperature. Cells were incubated in primary polyclonal antibodies (Bioss, Peking, China) for 1 h at 37°C. Antibodies examined included: CK3/CK12, CK18 and CK19, which were diluted with antibody diluents. The slide was gently shaken to remove the unbound antibodies and washed three times with PBS (5 min each). Then the cells were processed with polymer detection system for Immuno-Histological staining kit (PV-9000, Zhongshan Goldenbridge, Peking, China) to be incubated for 40 min at 37°C, and rinsed with PBS as described. Cells were colourated by DAB for 1 min. Finally, cell nucleus was counterstained with hematoxylin (Santa Cruz Biotechnology) for 5 min at room temperature. Imaging was performed using an invert microscope (Olympus) and a digital camera (Canon).

To induce hAECs into corneal epithelial-like cells, the cells were divided into two groups: the experiment group which hAECs were co-cultured with CECs, and the control group in which they were isolated and maintained in standard culture medium. Two steps were used: (i) Inducing culture: hAECs were seeded in the lower compartment of a two-chambered coculture well (Costar, 0.4 μm pore size, USA) at a cell density of 5×10^4/ml, and CECs were cultured at the same cell density in the upper compartment. Sterile glass slides were placed at the bottom of several lower compartments for scanning electron microscopy (SEM) detection. The culture plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. (ii) Culture after induction: After co-culture for a week, the medium was changed every 2 days. The CK3/CK12 and CK19 expressions in hAECs were detected by flow cytometry, and SEM was used for ultrastructural appearance.

CK3/CK12 and CK19 protein expression were detected to identify the hAECs. Meanwhile, their expression changes after co-culture were evaluated as the potential for the differentiation of hAECs into corneal epithelial-like cells. hAECs were detached from culture six-well plates with trypsin, washed and then permeabilized with 50 μl 0.1% Triton-X-100 for 5 min at room temperature. Cells were centrifuged at 1000 rpm for 4 min. After discarding the supernatant, cells were rinsed once with PBS. Cells were washed with PBS briefly and incubated with primary antibody (CK3/CK12, CK19) for 45 min at 4°C. Next, the cells were rinsed in PBS once again and incubated with the secondary antibody for 30 min at 37°C in the dark, and then at 4°C for 30 min. Add PBS to 500 μl. Cells were centrifuged and washed for once. Then 500 μl PBS containing 1% paraformaldehyde was added into the pellets and the cell solution was stored at 4°C in the dark until analysis. Cells were analysed by a flow cytometer (FACS Calibur, BD Biosciences, San Diego, USA). The data were analysed using Cell Quest software (BD Biosciences).

hAECs were fixed on coverslips in 2.5% glutaraldehyde and post-fixed in 2% osmium tetroxide. They were washed again in PBS before an alcohol series. Then the samples were dehydrated in a graded series of ethanol (50%, 80%, 90% and 100%), for 6 min respectively. They were transferred to hexamethyldisilazane (HMDS) (Sigma-Aldrich, USA) to remove residual water saturation for 2 min and air-dried for 2 h in a desiccator. Next, the samples were sputter-coated with gold for 90 sec, leaving an approximately 2 nm coating on the samples. The samples were examined using SEM (H7650, Hitachi, Japan).

Results are expressed as mean ± SE. Statistical significance of differences was evaluated by the matched *t*-test. Data were analysed using SPSS 17.0 software (SPSS Inc, Chicago, IL, USA). $P < 0.05$ was considered significant.

The first-passage CECs cultured using the biopsy-derived explant method showed good growth (Figure 1 *a*), exhibiting a round, oval or polygon shape. When cell proportion reached 80% of a well, the cells were digested with trypsin-EDTA, centrifuged, suspended with compound culture medium, and replated in a new six-well plate at a density of 1×10^5 cells per sq. cm. Medium was replaced 24 h after plating in order to remove unattached

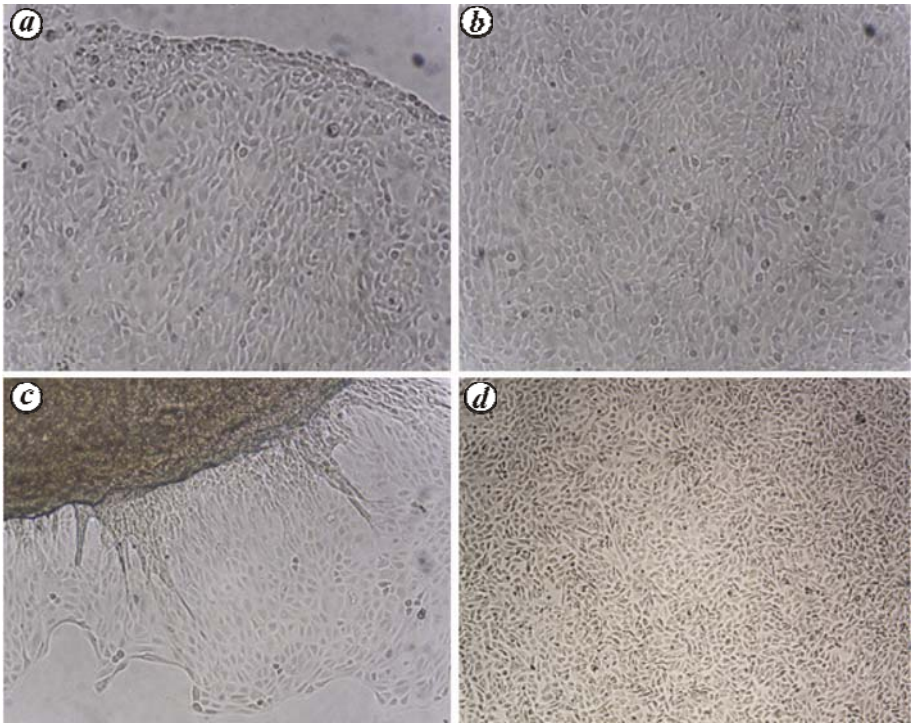

Figure 1. Morphology of corneal epithelial cells (CECs) and human amniotic epithelial cells (hAECs) cultured by tissue cultivation. ***a***, Primary CECs cultured by tissue cultivation. ***b***, The second-passage CECs fused on day-four. ***c***, Primary hAECs cultured on day-four. ***d***, The second-generation hAECs (magnification: 100×).

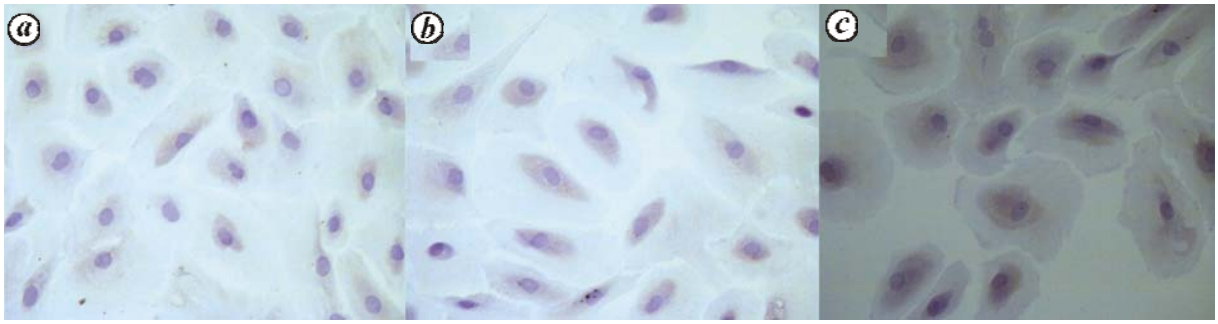

Figure 2. Immunocytochemistry staining of the second-generation hAECs. **a–*c***, The expressions of CK3/CK12, CK18, CK19 are positive in cytoplasm (magnification: 400 ×).

contaminating epithelial cells and then every 2 days. CECs appeared healthy being mostly hexagonal in shape with a fairly uniform size (Figure 1 *b*).

The growth process of hAECs was analogous to CECs (Figure 1 *c*). When hAECs reached 80% confluence, cells were digested and cultured in a new six-well plate at a density of 1×10^5 cells per sq. cm. The second-passage hAECs adhered to culture plates after 12 h and fused together at 3–4 days. hAECs by tissue cultivation exhibited an oval or polygon shape with large nuclei and uniform arrangement. They presented good refraction and favourable growing status with a plump nucleus, abundant cytoplasma, polygon shape and cell tight junctions (Figure 1 *d*).

hAECs possess high levels of CK3/CK12, CK18 and CK19 expression, and the protein expression was detected only in the cytoplasm on comparing with blank control group in which PBS was applied to replace the primary antibody. Cytoplasm presented a brownish-red colour, indicating positive staining. Figure 2 provides the results. Taken together, these observations strongly suggest the culturing of hAECs with tissue cultivation.

Figure 3 shows the positive CK3/CK12 and CK19 expressions in hAECs as detected by flow cytometry. The expression ratios of CK3/CK12 and CK19 in non-induced hAECs (control group) were 40.84% and 32.41% successively (Figure 3 *a* and *b*). However the induced hAECs showed higher average protein expression ratio

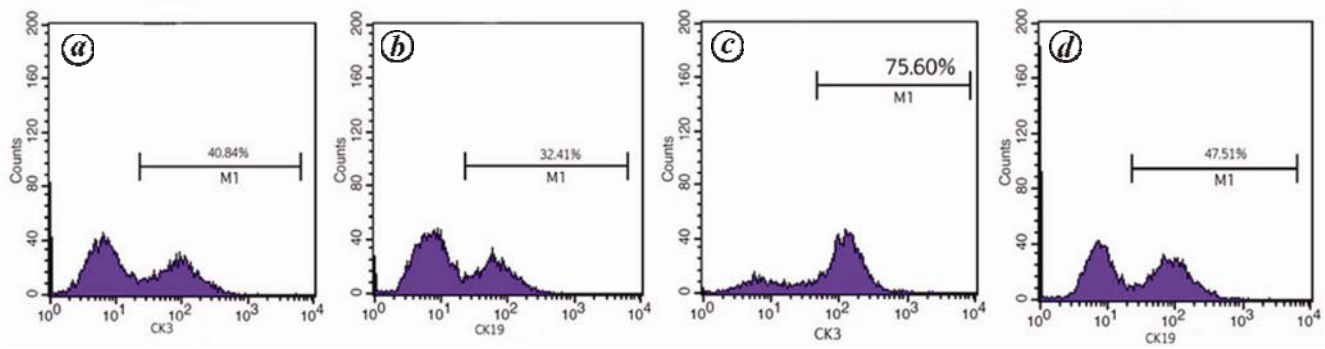

Figure 3. Flow cytometry analysis of hAECs. ***a***, ***b***, The average expression ratios of CK3/CK12 and CK19 on non-induced hAECs are 40.84% and 32.41%. ***c***, ***d***, For induced hAECs, the mean expression ratios of CK3/CK12 and CK19 are 75.60% and 47.51% respectively.

Table 1. Expression of CK3/CK12 and CK19 in primary cultured human amniotic epithelial cells (hAECs) assessed by flow cytometry

Marker	CK3	CK19
Non-induced hAECs (%)	41.82	33.40
Induced hAECs (%)	75.70	46.60

(CK3/CK12 75.60%, CK19 47.51%) than control group. This demonstrates that hAECs differentiate into corneal epithelial cells (Figure 3 *c* and *d*). Expressions of CK3/CK12 and CK19 were higher in introduced hAECs than non-introduced hAECs (Table 1). Statistical analysis recorded that there was significant difference between the induction and control groups ($P < 0.05$).

Before induction, hAECs were fat, long and spindle-shaped. The cells were closely attached to each other with distinct boundaries and extended transcellular strands. More microvilli and pseudopodia could be seen stretching from the cells. The diameter of the cells was about 60 μm (Figure 4 *a*).

After 1 week of co-culturing with CECs, the hAECs were observed to be uniform in size and shape, exhibiting applanation and polygon shape. The cells became thin and the intracytoplasm was reticulated; there were less microvilli and cell junctions on the surface of cells; pseudopodias were not seen. The diameter of hAECs was about 100 μm (Figure 4 *b*).

Corneal alkali burn is a serious clinical problem and can result in permanent visual impairment[12]. Recurrent epithelial erosions, corneal ulceration, severe stromal inflammation and neovascularization are common clinical consequences of alkali burn. The injury and defects of corneal epithelial layers are still important issues to be solved. Efforts to search for an adequate cell type and proper cell source have been conducted. A variety of cells derived from animal tissues are now used to replace CECs and reconstruct the corneal surface, such as bone marrow mesenchymal stem cells, human embryonic stem cells and orbital fat-derived stem cells[13–15].

HAECs are considered to be a promising cell source for their remarkable healing effect on corneal alkali burns. They can differentiate cell types of all three germ layers – ectoderm, mesoderm and endoderm[7,16–18]. Another reason for hAECs being ideal candidates for transplantation is that they do not express human leukocyte antigen markers under normal conditions. In other words, they are immunologically privileged cells[19]. These properties suggest that differentiated hAECs could modulate the inflammatory response of impaired tissue to improve prognosis potentially[20,21]. It has been demonstrated that hAECs can promote repair in animal models of acute lung injury[22,23]. It has also been shown that they secrete the necessary cytokines, growth factors, hormones and/or neurotransmitters[24] and etrophic factors, such as nerve growth factor[25] and novel epidermal growth-like factors[26] to restore cellular function. They also have a positive effect on regeneration of corneal epithelial cells in corneal alkli burn. hAECs have been utilized as feeder cells for limbal epithelial progenitor cells[27].

In this study, the phenotype of primary cultured hAECs was assessed by immunostaining with antibodies for specific markers such as CK18, CK3/CK12 and CK19 were detected by flow cytometry for quantitative analysis of cell phenotype. Immunostaining using anti CK3/CK12 labels, which are generally identified as the specific markers for mature cornea epithelial cells[28], allowed identification of differentiated cells on the amniotic epithelium. As a member of the cytokeratin family of intermediate filaments, CK19 has been suggested as a marker for the epidermal progenitors[29]. In this study, we observed that higher expressions of CK3/CK12 and CK19 were found in the induced hAECs than non-induced cells, which indicates that hAECs differentiate into the corneal epithelial-like cells.

SEM also proved that induced hAECs presented corneal epithelial-like cells features, including exhibiting applanation and polygon, uniform and larger size. The cell junction on the surface of cells was clear, suggesting that some hAECs had been induced to differentiate into corneal epithelial-like cells. We observed that almost all

of the hAECs showed morphological changes after co-culture. The factors that influence transdifferentiation require further studies.

Our findings represent a new paradigm for differentiation of hAECs into corneal epithelial-like cells to replace CECs. Under stimulation from CECs, hAECs acquire the characteristics of the former, including morphological changes and expression of CEC-specific proteins. Taken together, these observations provide evidence for differentiation of hAECs into corneal epithelial-like cells.

To summarize, the present study employed novel explant cultures to successfully cultivate hAECs, that was different from previous hAECs culture methods; the cultured cells presented benign and active state. Combining the higher levels of CK3/CK12 and CK19 expression detected by immunohistochemical assay with hAEC ultrastructure changes observed by SEM, we conclude that hAECs have the potential to differentiate into CECs. Collectively, hAECs possess the ability to differentiate into corneal epithelial-like cells to replace injured CECs. Their multipotential differentiation, anti-inflammatory, easy procurement and anti-angiogenic characteristics, make them an excellent choice for cell-based clinical therapy and future tissue engineering for corneal alkali burn. Although hAECs were successful in differentiating into corneal epithelial-like cells *in vitro*, there are several problems for utilizing these cells *in vivo*, such as cellular survival rate and instable phenotype after transplantation. Therefore, further research is needed to elucidate the main mechanisms before clinical applications can be considered.

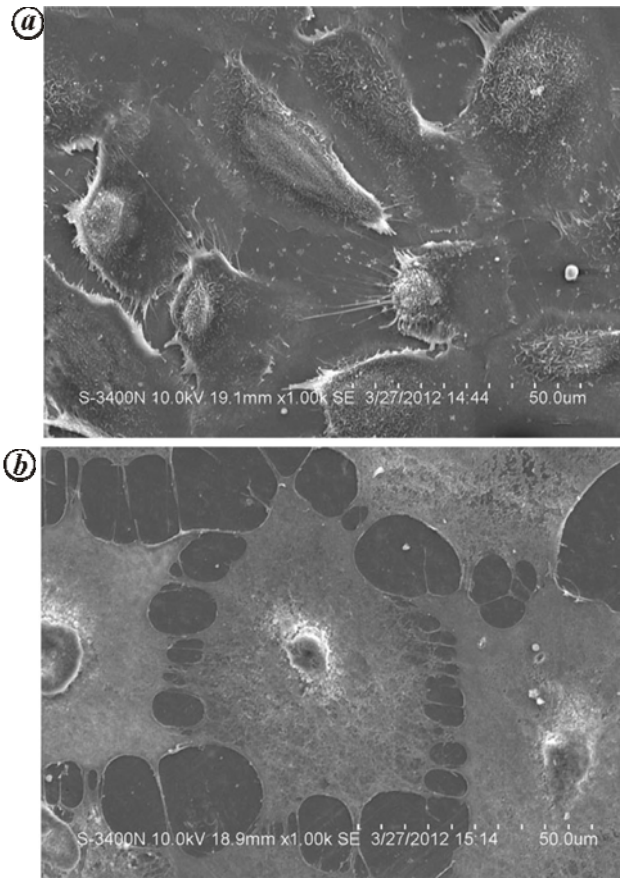

Figure 4. Ultrastructural characteristics of the hAECs. ***a***, Scanning electron microscopy of hAECs showing plump, long, spindle-shaped or polygonal appearance with some pseudopodias and several of microvilli. Diameter of the cells is about 60 μm. ***b***, Induced hAECs become thin, applanation and megagon with less microvilli and uniform in size and shape; and pseudopodias are not seen. Diameter of hAECs is about 100 μm (magnification: 1000×).

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Delineation of Trap and subtrappean Mesozoic sediments in Saurashtra peninsula, India

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Mapping of sediments beneath volcanic Traps is a highly challenging task. Here we report on the analysis of wide-angle seismic data from Trap-covered Saurashtra peninsula to address this problem. Travel-time modelling of mainly seismic refraction and some reflection phases yields basement configuration, trap and subtrappean sediment thicknesses along the Jodia–Ansador (NW–SE) profile in Saurashtra peninsula. Travel-time skip and amplitude decay in seismic refraction data indicate the presence of low-velocity sediments beneath the Traps. The result reveals two layers with Deccan Traps (4.85–5.0 km s^{-1}) followed by Mesozoic sediments above the basement (5.8–6.1 km s^{-1}). Using the lower bound velocity (3.2 km s^{-1}), sediment thickness varies between 800 and 1500 m. Based on upper bound velocity (4.3 km s^{-1}), we find both the sediment thickness and basement depth increase by 600–700 m. The thickness of sediments is more in the northwest and decreases gradually in the southeast, suggesting that the northwestern part of the profile is an important zone for hydrocarbon exploration in the Saurashtra peninsula. With the lower bound velocity of Mesozoics, we find that the basement (5.8–6.1 km s^{-1}) is deep (~2100 m) in the northwest and shallows up near Atkot to ~1.0 km depth, and then deepens further southeast, showing the basement upwarped. The overall velocity and boundary uncertainties are of the order of ±0.15 km s^{-1} and ± 0.15 km respectively.

Keywords: Seismic refraction, sediment thickness, travel-time inversion, volcanic traps.

THE Saurashtra peninsula is almost entirely covered by Deccan volcanics (Traps) with Lower Cretaceous (Mesozoic) sediments exposed in the northeastern part (Figure 1). Significant amount of Mesozoic sediments is believed to be hidden underneath the Deccan Traps. Oil industry has been engaged in exploring trap-covered regions for hydrocarbon potential, since Mesozoic sediments are the source rock for more than 50% hydrocarbon reserves world over[1]. In India, hydrocarbons have been discovered in Mesozoic sediments[2] in Jaisalmer basin of Rajasthan and East Godavari sub-basin of Andhra Pradesh. Presence of subtrappean Mesozoic sediments has been established through geophysical studies and drilling few bore wells in

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