Preclinical Investigation of Pre-malignant Oral Fibrosis and Carcinoma in Lagomorphs

Pradip Kumar Majumdar[a], Pritam Sukul[b], Sandhini Saha[c], Rohit Verma[c*], Supriya Sen Gupta[d], Supriya Nath[e], Bijan Basak[f], Anuj Tripathi[g]

[a]Head of the Department of Surgery (MS, FIAS), JN Ray General Hospital, Kolkata, India.

[b]Scientist (Marie-Curie Fellow, PIMMS EU-Network) at ROMBAT Research Group, Dpt. of Anesthesiology and Intensive Care Medicine, University of Rostock, Germany.

[c]Applied Microbiology, Department of Botany, Banaras Hindu University, India.

[d]Head of the Post-Graduate Department of Zoology, Asutosh College, University of Calcutta, India.

[e]Department of Surgery, JN Ray General Hospital, Kolkata, India.

[f]Department of Pathology, Calcutta Medical College, India.

[g]CEO, Clini India[TM], India.

Abstract: ***Pre-malignant oral fibrosis carcinoma (PMOFC) is a potentially severe condition of the Submucous layer of oral cavity, which is characterized by juxtra epithelial inflammation and progressive fibrosis at lamina propria as well as in deeper connective tissues of the oral cavity including oropharynx and upper third of the esophagus. A number of epidemiological surveys, case-series reports, large sized cross sectional surveys, case-control studies, cohort and intervention studies have provided an over whelming evidence that Areca nut+tobacco are the main etiological factor for PMOFC. Experimental evidence to establish the above proof of concept still lies on analytical challenges and limitations. The objective of the present study was to implement an in vivo model that has shown characteristic features of collagen for PMOFC in relation to fibrosis promoting activity of areca nut+tobacco combination in Lagomorphs (eg. Oryctologous sp.). The method used for study includes Areca nut+tobacco (experimental extract), o-phenyl phenol (positive control) and normal saline (negative control) were administered in the lower jaw of Lagomorphs (three groups) with 6 days of interval for nine months. Protein modification and DNA damage caused by Areca nut+tobacco in oral tissue and blood were analyzed by MALDI-TOF and comet assay respectively. It was found that the oral epithelium, treated with Areca nut+tobacco has shown progressive changes in thickness leading to palpation, ulceration, irregular growth and restricted mouth opening. DNA damage in blood was found by comet assay. Tissue protein modification was established by MALDI-TOF with the help of SDS-PAGE, 2D-Gel electrophoresis and tryptic gel digestion. The study concludes stating that the model has the potential to compare the effect of Areca nut+tobacco extract against other standard carcinogen (o-phenyl phenol) in presence of a negative control through collagen modification and DNA damage. Thus this study provides further evidence that the experimental extract significantly contributes to the establishment of PMOFC in treated organisms.***

Keywords: Areca nut, collagen, Lagomorphs model, premalignant oral carcinoma, tobacco.

1. Introduction

Pre-malignant oral fibrosis carcinoma is regarded as a submucous fibrosis [1], which is a precancerous condition of oral subepithelial connective tissue resulting in stiffness of the oral mucosa that causes inability to open mouth. PMOFC is histologically characterized by epithelial atrophy and progressive accumulation of collagen fibers in the lamina propria and submucous layer of the oral mucosa [2].

The hallmark of the disease is submucosal fibrosis that affects the oral cavity and progressively involves the pharynx and the upper esophagus. It is characterized by juxta-epithelial inflammatory reaction followed by chronic change in fibro-elasticity of the lamina propria that is associated with epithelial atrophy. These lead to burning sensation in oral cavity, blanching /stiffening of oral mucosa and oropharynx, resulting in restricted mouth opening, which in turn causes limited food consumption, difficulty in maintaining oral health as well as impairs ability to speak [3–8]. The signs and symptoms depend on the progression of lesions and number of affected sites [9]. What makes it more sinister is its malignant transformation rate, which has been reported to be around 7.6% over a 17-year period [10–14].

Pre-malignant oral fibrosis is predominantly found among the people of South Asia that is closely associated with the habit of betel-quid with tobacco chewing. Several case–control studies have shown evidences that areca nut, a constituent of betel quid is the major risk factor for oral cancer. Betel quid is a combination of betel leaf, areca nut, and slaked lime. In many countries, tobacco is also added, and the product is known as *gutka*, *ghutka*, or *gutkha*[15]. Another one is mawa, that prepared from sun-cured areca nut and mixed with tobacco flakes and slaked lime. Areca nut is chewed in various forms: raw, boiled or roasted. There are approximately 600 million people worldwide (10–20% of the global population) who use raw or any processed form areca as a chewing substance [16].

A variety of etiologic substance and factors such as capsaicin, betel nut alkaloids, tobacco, hypersensitivity, autoimmunity, genetic predisposition and chronic iron and vitamin B-complex deficiency have been suggested by various authors, [17-21] the most common of which are chewing areca nut+tobacco combination. Excessive use of areca nut may cause fibrosis carcinoma due to increased collagen synthesis and thus induce the production of free radicals and reactive oxygen species. These are responsible for increased oxidation/peroxidation of polyunsaturated fatty acids, which affect essential constituents of cell membrane and might be involved in tumor-genesis. [22, 23]

Areca nut is an endosperm of a fruit from *Areca catechu*, which has carbohydrates, fats, proteins, crude fiber, polyphenols (flavonoids and tannins), various alkaloids and mineral matter as its major constituents [24]. While chewed, alkaloids (such as arecoline, arecaidine, guvacine and guvacoline) are released from areca nut as the most important chemical components that produce a range of physiological effects on cardiovascular and neurological systems [25-27]. These alkaloids have powerful parasympathetic properties, which produce euphoria and counteract fatigue.

Nitrosation of arecoline leads to the formation of areca nut specific nitrosamine namely nitrosoguvacoline, nitrosoguvacine and 3-methylnitrosominopropionitrile, which alkylate the DNA. Metabolism of these areca nut specific nitrosamine leads to the formation of cyanoethyl group, which binds with o'methyl guanine in DNA. Prolonged exposure to these agents leads to malignant transformation.

About 35–40% of tobacco consumption in India is in smokeless forms, mostly of the species *Nicotiana rustica* that have been found to contain high concentrations of tobacco-specific nitrosamines including nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3pyridyl)-1-butanone (NNK), N' -nitrosoanatabine (NAT) and N' nitrosoanabasine (NAB). However tobacco as an ingredient in some areca nut mixtures is not a causative factor for PMOFC, but is responsible for a higher occurrence of PMOFC due to increased addiction and concurrent use of areca nut [28].

In habitual betel quid tobacco chewers, premalignant oral carcinoma may caused by the amount of tannic acid content in the betel nut, the influence of mixed calcium powder and the conditional action of arecoline content in betel nut that affect the vascular supply of oral mucosa and cause neurotropic disorder. Further addition of slaked lime [Ca (OH) 2] to areca nut with tobacco in pan facilitates hydrolysis of arecoline to arecaidine (more potent) making this agent available in the oral environment.

The extracellular matrix (ECM) is a dynamic, intricate network of macro molecules that plays an important role to regulate cellular function during normal and pathological remodeling processes, such as inflammation, tissue repair and tumor development [29]. The major components of ECM are proteoglycans, collagens and glycoproteins, and the structural features of these components provide the basis for their involvement in a variety of interactions following cellular signaling [30]. PMOFC has been described as a disorder of collagen metabolism in which there is an increase in production of highly cross-linked insoluble collagen type 1[31]. Cross linking of collagen plays a vital role in fibrosis. Fibrosis of the subepithelial connective tissue in PMOFC has been identified as the patho-genomic feature which gives rise to limited mouth opening in human[32,33]. It differs from pathological fibrotic conditions such as scleroderma, juvenile aggressive fibromatoses and abdominal desmoids [34] however, the nature and type of fibrosis in PMOFC have not yet been morphologically characterized. An electron microscopic study by van Wyk *et al* [35] showed no abnormal collagen in PMOFC. Concerning the type of collagen involvement in PMOFC, whether it consists of specific subtypes or a mixture of collagen has not yet been resolved [31, 35, 36]. Several studies have been carried out to elucidate the mechanisms underlying excessive deposition of collagen in the lamina propria in PMOFC patients [37-39]. Based on the molecular pathogenesis, PMOFC has been described as a collagen metabolism disorder due to the presence of a key enzyme lysyl oxidase that tilts the balance of collagen metabolism towards fibrosis [40].

Flavanoid, catechin, and tannin in betel nuts let collagen fibers to cross-link, making them less susceptible to collagenase degradation [41]. This results in increased fibrosis by causing both increased collagen production and decreased collagen breakdown. Pre-malignant oral fibrosis carcinoma remains active even after cessation of the chewing habit, suggesting that components of the areca nut with tobacco initiate oral submucous fibrosis and then affect gene expression in the fibroblasts, which then produce greater amounts of normal collagen [42]. Chewing areca quid may also activate NF-kappaB expression, thereby stimulating collagen fibroblasts and leading to further fibrosis in persons with oral submucous fibrosis [43]. Studies have shown that there is 1.5 fold increases in collagen production by PMOFC fibroblast and with the progression of disease type 3 collagen is completely replaced by type 1 collagen which is more resistant to degradation. Also there has been an excess of alpha 1(1) chain relative to alpha 2(1) chain, suggesting an alteration of collagen molecule during the disease progression.

Areca nuts have also been shown to have a high copper content, and chewing areca nut with tobacco for 5-30 minutes significantly increases soluble copper levels in oral fluids. This increased level of soluble copper supports the hypothesis that copper acts as an initiating factor in persons with oral submucous fibrosis by stimulating fibrogenesis through up-regulation of copper-dependent lysyl oxidase activity[44]. Further, a significant gradual increase in serum copper levels from precancer to cancer patients has been documented, which may have a role in oral fibrosis to cancer pathogenesis [45].

There are no established animal models of PMOFC or oral fibrosis till date [23]. However, Huang *et al* [46] claimed to have produced a rat model of submucous fibrosis in Hunan Medical University, China and the in vivo experiments of Khrime *et al* [47] showed histopathological findings akin to submucous fibrosis induced by pan masala on the rat mucosa. The characterizations of these models are not completed and the experimental evidence is neither convincing nor reproducible. The aim of this study is to demonstrate the

potential contribution of Areca nut+tobacco extract in the investigation of PMOFC in treated Lagomorphss.

2. Materials and Methods

To investigate the pre-malignant oral fibrosis carcinoma in animal model we choose a combination of Areca nut+tobacco extract solution as a compound for treatment. The aqueous solution of Areca nut+tobacco extract was prepared from fresh, mature endosperms of *A. catechu* and dried leaf of tobacco by dissolving in milli-Q water. To get the experimental extract, at first crashed the Areca nut and tobacco by mortar pistol, then take 1.5gm of areca powder and 0.5gm tobacco powder with 5ml of water and extract is collected from the solution by precipating the solid portion by whatman filter paper.

For the investigation of premalignant oral carcinoma we selected three groups of Lagomorphs. One group as negative control {administered with normal saline}, 2nd group as positive control {treated with *o*-phenyl phenol} 3rd group treated with experimental combination (areca and tobacco extract). We injected these three solutions (1ml of each) separately into the lower jaws of those Lagomorphs maintaining a six days of interval for three months. The complete process was repeated for another 6 months.

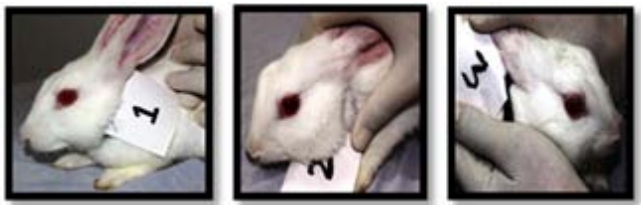


Figure 1: Showing one individual from each of the three groups of lagomorphs

Figure 2: Admistration of experimental extract

To see the DNA damage in each group of Lagomorphs, blood samples were collected from those individuals for three times in two months of interval. Around 5ml of blood was collected from each individual in tubes using sterile disposable syringe. In each tube a proportionate amount of EGTA was added to prevent the blood from clotting.

To analyze the DNA damage in blood we used single cell gel electrophoresis (comet assay), which involves the encapsulation of cells in a low melting point. Agarose suspension lyses the cells in neutral or alkaline conditions, and after words electrophoresis was performed on the suspended lysed cells. Subsequently the whole genomic DNA was analyzed by using a similar approach of agarose gel electrophoresis.

To see the protein modification we had collected the tissues samples from the lower jaw of each individual through punch-biopsy and stored those samples in 25mM Tris–buffer (pH 9.5) at -20^{0}C.

Proteins were extracted from tissue samples according to our custom made protocol. The tissues were washed and homogenized in 25mM Tris-buffer (alkaline pH) containing 1M/L PMSF by electric homogenizer. The mixture was centrifuged upto 5500×g for 17mins to remove cellular debris. The Homogenate solution was again centrifuged at 15050rpm for 35mins at 4^{0}C and the supernatant was collected as whole tissue protein was now in the same. After collecting the protein we added 6µl of protein inhibitor in each tube. Finally, these proteins were estimated by the Bradford method.

In the next stage the estimated protein mixtures were loaded in SDS-PAGE considering same amount and further clarification of each protein was subjected by a 2D gel electrophoresis. Then these gels were stained by the colloidal Coomassie-Brilliant-Blue and silver nitrate (increase specificity). Each and every protein was extracted from the gel band by the tryptic in-gel digestion.

The dried extract will mixed with in 10µl of 0.1% TFA. A reverse-phase ZipTipC18 micro-column (ZipTip is a 10 µL pipette tip with a 0.6 or 0.2µL bed of chromatography media fixed at its end with no dead volume. It is ideal for concentrating and purifying samples for sensitive downstream analyses) was pre-estimated with 55% acetonitrile and wash with TFA. Peptide binding was facilitated on the reconstituted sample within the tip. To avoid further contamination 12µl of TFA (0.1%) was used for washing (four times). Finally MALDI sample plates were prepared directly after the illusion of peptides in 55% acetonitrile (at 0.1% TFA buffer).

MALDI-TOF analysis was performed after the crystallization (at NTP) of spotted sample plates after the addition of CHCA in presence of equal volume of acetonitrile and TFA buffer.

3. Results

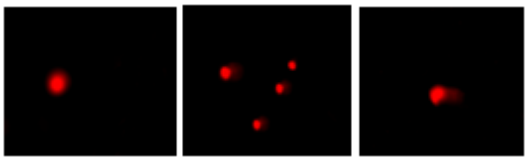
After each three months (9 months in total) of administration finally we have successfully implemented pre-malignant oral fibrosis/carcinoma (at oral submucous region) in Areca nut+tobacco treated Lagomorphs whereas oral cancer was observed in the positive control group.

General observations:

[A] [B]

Figure 3: White irregular growth of subepithelial in *O*-phenylphenol treated rabbit [A] and ulceration involved in jaw angle of the arecanut+tobacco experimental extract treated Lagomorphs [B]

Trough out the investigation period, any significant morphological deformities were not observed but some noticeable loss of body weight in experimental extract and o-phenyl phenol treated Lagomorphs. In case of experimental extract treated Lagomorphs a distinct sing of restricted mouth opening because of uncontroled growth and palpation in indurated on margin of oral cavity. Ulceration was manifested predominantly, including half of the lower jaw. The extent of DNA damage was presented (proportionally) by the 'length of tail', that was found in comet assay.



A **B** C

Figure 4: Comet images shows the assay administered with normal saline [A], Areca nut+tobacco [B] and o-phenyl phenol [C]

Table 1: Quantitative representation of DNA damage

Sample	*DNA tail length(µm)*
Control (normal saline)	2.3
Positive control(o-phenylphenol)	5
Areca nut and tobacco	3.68

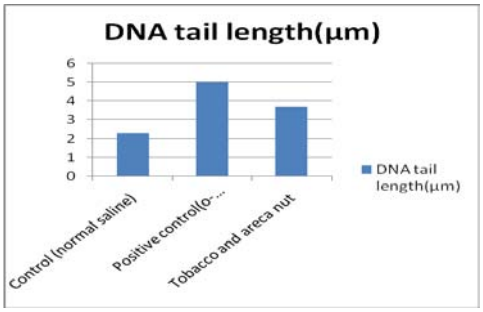
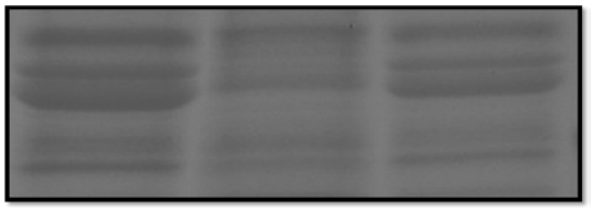


Figure 5: Graphical presentation of DNA damage by comet assay

From the standard BSA graph, we have measured the amount of proteins. The concentrations of those proteins were 1.98µg/µl, 1.39µg/µl and 0.99µg/µl restively for normal saline, positive control and treated Lagomorphs. Finally 45 µg of each protein was loaded in SDS-PAGE just after dissolving those proteins in 350µl of Tris buffer. After that 350µl of protein was loaded in an IPG strips for 2D Gel Electrophoresis followed by the respective gel pictures obtained from SDS-PAGE experiments.



A B C

Figure 6: Lane A: The protein bands from Positive Control (*O*-phenyl phenol) tissue
Lane B: The protein bands from Control (normal saline) tissue
Lane C: The protein bands from Treated (Areca nut and tobacco) tissue

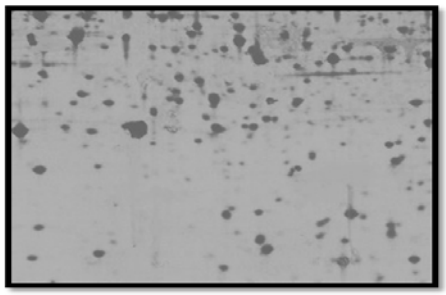


Figure 7: Two Dimensional (2D) Gel picture of Areca nut+tobacco treated tissue protein.

From this stained gel, we have got different spots of proteins at various isoelectric point (pI). After that we have implied Tryptic in-gel digestion Kit on the proteins bands which were sampled from those gels. Finally the subsequent proteins were analyzed in MALDI-TOF.

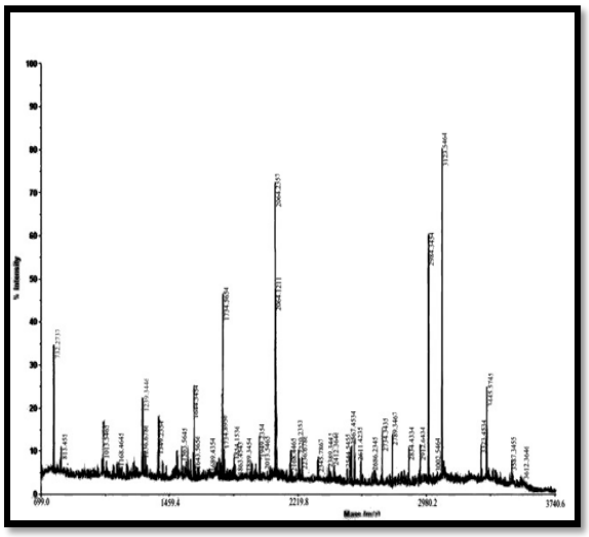


Figure 8: Tryptic MALDI-TOF spectrum of Collagen type 1 (COL1) precursor protein.

1 WERTXCDEXCXWFRGARGPPGAVGSPGREWTHR
TVNGAPGEAGRDGNPGS
51 DGPPGRDGQPGHKGERGYPGNAGPVGAAGAPGP
QGSVSDWSCRWEEWERF
101 EREWGFGFDFGHGPTGKHGNRGEPGPAGSIGP
VGAAGPRGPSGPQGIRGD
151 KGEPGDKGPRGLPGIKGHNGLQGLPGLAGQHG
DQGAPGAVGPAGPRGPA
201 GPASFWFFWFDWESDGFFDVEFTGPAGKDGRS

GHPGTVGPAGLRGSQGS
251 QGPAGPPGPPGPPGPPGASGGGYDFGYDGDF
YRADQPRSPPSLRPKDYE
301
VDATLKSLNNQIETLLTPEGSRKNGHHJFGDFFSDV
FGPARTCRDLRLSH
351 PEWSSGYYWIDPNQGCTMDAIKVYCDFSTGETC
IRAQPENISVKNWYKS
401 SKAKKHVWLGESRTYYRWGVTINGGTQFEYNV
EGVTSKEMATQLAFMRLL
451
FHFDDSGFDFDVFHNSDSDFGYBZKSAGVSVPGPM
GPSGPRGLPGPPGAP
501 GPZGFZGPPGZPGZPGSSANHASQNITYHCKNSIAY
MDEETGNLNKAVIL
551 QGSNDVELVAEGNSRFTYTVLVDGCTKK

Figure 9: COL1 coverage map. Matched peptides shown in **Bold Red**

4. Conclusion

It is generally observed that the onset of PMOFC in human is insidious and takes about 2–20 years to manifest with symptoms. Submucosal carcinoma in different areas of oral mucosa leads to difficulty of mouth opening process (trismus), while blanching of the oral mucosa is an important clinical feature at an early stage of PMOFC [48,49].

Epidemiological studies have established a strong causal relationship between the use of Areca nut+tobacco with that of PMOFC prognosis [50,51]. Population that is exposed to Areca nut+tobacco have an odd ratio ranging from 60 to 132, relevant to the occurrence of PMOFC, compared with non-users [50,52]. Evidence of genotoxicity of areca nut has been recently revealed [24]. Lagomorphs that are exposed to aqueous extract of areca nut with tobaccos, have shown to implement of PMOFC in the lower jaw [53, 54]. There has not been any reproducible animal model of PMOFC to the best of our knowledge. Non-availability of an animal model of PMOFC limits further research into the understanding of the pathogenesis of this disease and furthermore, development of therapeutic agents to control the progression of this disease [55]. Therefore, the present study was undertaken to investigation of PMOFC in Lagomorphs as well as to compare that with both positive and negative control by comet assay and MALDI-TOF to show DNA damage and protein profile respectively.

Microscopically it is observed that unlike humans, Lagomorphs have a sparsely distributed lamina propria below the epithelium. This morphological feature could be an added advantage, especially in relation to PMOFC. As a result, at the onset of fibrosis, the extension of collagen fibers deep into the tissues could affect striated muscles underneath. This phenomenon has important considerations to the Lagomorphs described here. The minimum size of the oral cavity is a distinct advantage in monitoring clinical changes by visual investigation and large amount of tissues we were collected which is helpful for MALDI-TOF.
The present study was able to establish the DNA damage and collagen deposition in the buccal mucosa epithelium in all the experimentally treated Lagomorphs at all treatment periods (Fig.3). The present study shows some irregular growth in the lower jaw and white colored subepithelial buccal mucosal tissues of treated Lagomorphs (Fig.3).

From the above results it could easily concluded that the study was performed to obtain the change in protein and DNA damage in blood of the Lagomorphs after the exposure of Areca nut+tobacco extract and o-phenyl phenol. As areca nut is a causative agent for the PMOFC and tobacco contains the carcinogenic and mutagenic compound, thus we selected Areca nut+tobacco as an experimental extract and we also treated one group of Lagomorphs by *o*-phenyl phenol that was acted as positive control. Finally we have monitored the PMOFC (ulceration, irregular growth and palpation) in the lower jaw of the treated Lagomorphs.

From those infected areas we have collected the tissue and blood samples from each group of Lagomorphs. We have seen DNA damage the blood of the treated and positive controlled Lagomorphs by the comet assay. Figure 3 have clearly demonstrated more amount of DNA damage for positive control than in the treated group.

We have successfully identified collagen type 1 protein among the results obtained from MALDI-TOF. These collagen are predicted to be associated with the occurrence of premalignant oral fibrosis and carcinoma. Additional modification of collagen protein is also found in treated Lagomorphs which was absent in negative control group.

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Author Profile

Dr. Pradip Kumar Majumdar, (MBBS, MS, FIAS) He is the Head of the Department of Surgery at JN-Roy Sishu Seva Bhavan and General Hospital, Kolkata, India.

Pritam Sukul Scientist (Marie-Curie Fellow, EU-PIMMS Network) at ROMBAT Research Group, Department of Anesthesiology and Intensive Care Medicine, University of Rostock, Germany. Previous: Post-Graduate Research and MSc in Clinical Research at Cranfield Health, Cranfield University, UK. He completed BLS & ACLS from American Heart Association. Foundation Clinical Research and PGDPM, ICRI, India, BSc Zoology (HONS) University of Calcutta. Three years of experience in Cancer Research in India.

Mr. Rohit Verma has obtained MSc Applied microbiology from Banaras Hindu University, UP, India, BSc Biotechnology from Chhatrapati Sahu Ji Maharaj University U.P, India. He has subsequent experience in Bio-Medical Research in India. Completed *BLS & ACLS from American Heart Association*

Miss. Sandhini Saha has obtained MSc Applied Microbiology from Banaras Hindu University, UP, India. BSc Microbiology (Position: First Class First) from Kalyani University, WB, India. She has subsequent experience in Bio-Medical Research in India. Completed *BLS & ACLS from American Heart Association*

Dr. Supriya Sengupta: Associate Professor and Head at Post-Graduate Department of Zoology, Asutosh College. She obtained BSc and MSc in Zoology (HONS). She is the recipient of National Scholarship. She has teaching experience of Sixteen years. She has worked as CSIR Junior and Senior Research Fellow for five years. Fellow/Member of Learned: LM: ZS, LM: BBP.

Dr. Bijan Basak, Post Graduate Trainee (MD) in Pathology. Department of Pathology, Medical College, Kolkata, INDIA. M.B; B.S (Year- 2009) From Medical College, Kolkata, the West Bengal University of Health Sciences. Working Experiences: Junior Resident, Department of Neuro-medicine, Medical College, Kolkata for 1 year & 6 months.

Dr. Supriya Nath Doctor in J N Roy Sishu Seva Bhavan and General Hospital, Kolkata, India.

Mr. Anuj Tripathi CEO of Clini India™, India. He has obtained B.Sc. and M.Sc. in Biotechnology and Post Graduate Diploma In CA from C.S.J.M. University, Kanpur. He has 7.5 years of diverse experience in Life Science, Pharmaceutical & Clinical Research Segment.