DISCUSSION

For past several decades, it was believed that bone is an inert connective tissue. However, this notion has changed with time, now bone is considered a dynamic endocrine organ (68). OI is a genetic bone disorder characterized by low bone mass and increased bone fragility. Bone histomorphometry suggested that OI patients have impaired osteoblastic activity, decreased bone formation and mineral content at single cell level (158). The coiled-coil triple helical structure of type I collagen was first proposed by Ramachandran et al. (89, 159). Triple helix is formed by three polypeptide chains in which glycine are obligatory residues at every third position for a particular type of the molecular packing of collagen fibers (33, 88). Type I collagen is synthesised as procollagen molecule consisting of N-terminal propeptide and C-terminal propeptide centre with helical domain containing three major ligand-binding regions (MLBR) relevant for interactions with intercellular proteins. The substitution of glycine by any other charge residue leads to weak hydrogen bonding, structural clashes and instability in the collagen triple helix (35). OI is a heterogeneous group of bone disorders and discovery of several new causative gene mutations (autosomal dominant as well as recessive) has prompted a substantial amount of modification in its existing genetic classification of the disease (43).

Molecular understanding of OI has led to two approaches of classification a) genetic–clinical, under Sillence classification and b) genetic-functional, under-identification of genes without clinical correlation (160, 161). Majority of the OI cases have been reported with structural and quantitative mutations in type I collagen gene while other genes related to processing, folding, cross-linking and secretion of procollagen have minimal role (151). Due to variability in gene mutation and its association with OI expression, more comprehensive studies are required to explain the pathogenicity of the disorder.

In our study OI patients with type I and IV showed relatively mild phenotype whereas, types II and III showed severe clinical expression. Majority of OI patient had the onset of fracture at birth similar to other reports (162). Whereas, none of our OI patients had hearing loss but scoliosis and hyperextensibility of joints are more commonly seen in severe forms (II and III) of OI as also observed by other groups

(163). Another study from north India with 20 subjects showed dentinogenesis imperfecta, blue sclera in 50% of the cases and fracture in all cases, which is similar to our study (46). For genotype-phenotype correlation molecular and structure analysis were performed. We found 16 variants of type I collagen gene and one of SERPINH1 gene. We observed that some OI patients had defect in collagen synthesis, the mutations in exon 34 and 50 of COL1A1 gene; frameshift 34c.2321delC, p.Gly774Leu, premature stop codon c.4021C>T, p.Gln1341X. This mutation resulted in defective collagen which altered the normal collagen functioning, the mechanism is known as procollagen suicide. The structural alteration in the triple helix interferes with normal processing of the procollagen due to silent mutation (c.1209 T > A, p.Pro403Pro and c.3702 C > T, p.Thr1234Thr) in exon 19 and 48 respectively. Additionally, the premature stop codon in exon19 c.1243C>T, p.Arg415X and missense mutation in exon48 c.3556 C>G, p.Pro1186Ala; mutation in MLBR region are exclusively lethal which indicates the interactions between non-collagenous matrix proteins and the collagen monomer of COL1A1 gene. First five exons encode N-terminal propeptide region and missense mutation in exon1 c.97 G > A, p.Asp33Asn; premature stop codon in exon2 c.141C>A, p.Tyr47X, a missense mutation in exon2 c.182G>T, p.Cys61Phe, silent mutation in exon3 c.87 T > C, p.Thr29Thr and exon5 c.391C>T, p.Arg131X of COL1A1 gene. The triple helical domain consists of exon16 c.1056 G>A, p.Gly352Ser, exon17 c.435 G>A, p. Gly145Ser, exon25 c.495 C >T, p. Gly165Ser, silent mutation in exon33 c.623 T >A, p.Pro403Pro, substitution mutation exon49 c.3304 C >T, p.Gly110Cys.

Procollagen reaches to ER lumen where post-translation modification occurs in chain of abnormal procollagen, which is retained in the lumen and get exposed for long time for modifications. Triple helix procollagen needs HSP47 molecules for stability, and in our study we found that mutation in the SERPINH1 gene is a missense mutation in exon1 c.233 T>C, p.Leu78Pro. To correlate this mutation with structural alteration, computational analysis was performed. A heterozygous missense mutation in exon 49 of COL1A2 gene (chr7:94056975; G>G/T); which results in substitution cysteine to glycine at codon 1102 (p.G1102C; ENST00000297268). We performed in silico mutagenesis and the PDB structure 5CTD was selected as templet. As previous reviews suggested that only one mutation is required in chain B of COL1A2, to develop OI. We found that mutant structure is highly unstable and sterically disrupts

the type I collagen structure. To understand the cellular importance and role of HSP47 in OI patients, functional studies were performed (164, 165). In this study, we have shown that mutation in HSP47 leads to reduced expression and accumulation of the type I collagen in the ER, resulting in ER stress. Other studies have shown that ER stress leads to procollagen misfolding resulting in severe form of OI (29). On the basis of above findings, we propose the role of HSP47 in the synthesis of type I collagen. We suggest that HSP47 can be used as a diagnostic and therapeutic target for OI. Currently, BP's are first line of treatment for OI, they. BPs increase the bone mass and reduce the number of fractures in OI patients. We have observed that high dose (>30mg) of ZOL acid causes suppression of bone turnover markers and osteosclerosis of bones. We also observed the presence of dense metaphyses in long bones (**Fig12e)** in the subjects who received high doses of BPs. The recommended dose of ZOL for paediatric group is 0.05mg per kg body weight, every six monthly (45). However, our patients received (mean dose) 16 mg per year. Prolonged use of ZOL suppresses bone turnover markers (PINP & CTx) (166) we have also observed similar findings in our OI patients. Regarding safety concern, residual BPs levels are measured in growing skeleton after the therapy. Our study participants received more than five times of ZOL usually prescribed in the pediatric population. Though the metaphyseal sclerotic "banding" was seen on cyclic BPs therapy, it may be resolved after completion of treatment. However, it persisted in our patients even after cessation of treatment.

We observed significantly high Caspase-3 activity which is a marker of apoptosis, in osteoclasts isolated from PMBCs of ZOL (>30 mg) treated OI children. Hughes et al. found 4-24 fold increase in the proportion of apoptotic osteoclasts after the treatment with BPs (risedronate, pamidronate, and clodronate) in vitro. Among the three bisphosphonate compounds, risedronate, the most potent inhibitor of bone resorption in vivo, was the most reliable inducer of osteoclast apoptosis in vitro (167). In another study by Rogers et al., it was found that bisphosphonates induces osteoclast apoptosis, in part by inhibiting the enzyme activity in the mevalonate pathway and by promoting caspase cleavage of mammalian sterile 20-like (Mst) kinase 1 (168). Since in healthy condition of bone remodeling, bone resorption and formation are coupled to each other, this osteoclast apoptosis is expected to decrease bone formation. This kind of "acquired osteopetrosis" has been previously reported by Whyte et al in a child of OI

treated with pamidronate (169). Recent studies suggests a fundamentally different mechanism of action of BPs and rhPTH at the tissue level; however, the molecular basis is not explored. Therefore, in the current study, we studied the effects of zoledronic acid (ZOL; at 1µM and 5µM) and rhPTH (at 5µg and 10µg) on U2OS cells. Cellular viability, mineralization, and osteogenic gene expressions were assessed to elucidate the effects of these two prototypic drugs with diametrically different mechanisms of action. We observed that cellular viability was not affected by either ZOL or rhPTH treatment alone or in sequential therapies. Osteoblastic activity (alkaline phosphatase activity and mineralization) increased significantly with rhPTH followed by ZOL. Alkaline phosphatase gene activity increased considerably with sequential treatment with rhPTH followed by ZOL both at the mRNA and protein levels. Also, other osteoblastic genes (COL1A1 and osteocalcin) were significantly modulated by sequential treatment with rhPTH followed by ZOL. We conclude that 1µM of ZOL or 5µg of rhPTH alone or in sequential treatment rhPTH followed by ZOL showed the best anabolic effects**.** We found that both ZOL and rhPTH had distinguishable effects on osteoblast cell viability, mineralization, and osteoblast ALP activity as well as on osteogenic expressions (**Figures 35-38**), similar to other findings (53, 170, 171). Whereas one study (172) is not in agreement with our findings. It may be related to the type of cell lines, the dose and class of drugs used, nevertheless, the directional changes were similar to previous studies (53, 153, 157). We have shown the effect of sequential treatments on osteoblast cell viability, function, and gene expressions for the first time (173, 174).

The BPs exert their anti-fracture efficacy primarily by promoting osteoclast apoptosis, thus inhibiting bone resorption (175). However, both in vivo and in vitro studies suggest that BPs may have osteoanabolic effects (176). It has been suggested that at nM concentrations BPs may have an osteoanabolic effect (177), and in 1µM concentrations, BPs exert a dose-dependent negative effect (178, 179). A similar dose-dependent differential effect of BPs on osteoclasts and osteoblasts might occur in humans, however there is no evidence yet. Our studies with ZOL alone or in sequential treatments (ZOL followed by rhPTH or rhPTH followed by ZOL) suggest that at 1µM level ZOL alone or ZOL followed by rhPTH decrease ALP activity and osteogenic gene expressions. In contrast, rhPTH followed by ZOL either had no effect or a slight increase in these effects which suggest that sequential treatments should

always be initiated with rhPTH first. These findings are consistent with the clinical observations that BPs might blunt the anabolic effects of teriparatide in patients with post-menopausal osteoporosis if given after prolonged BP therapy (180-182). Role of rhPTH as a potent anabolic and ZOL a strong prototypical anti-catabolic agent have been used widely for the treatment of osteoporosis and other metabolic bone disorders (10, 181-183). A recent study on bone biopsy have demonstrated the differential mechanism of actions of rhPTH and ZOL both at the systemic (as assessed by bone turnover markers) as well as at the tissue level (as determined by bone histomorphometry) (184), but the molecular basis for these observations not explored (10, 181, 183).

For further validation of the mutants are required for loss or gain function of the gene. The small sample size for evaluating ZOL concentrations on osteoclast precursor cells. Osteoblastic cell lines may not apply to the clinical practice, we only used a fixed-dose (5µg rhPTH and 1µM ZOL) model based on previous and our dose-response studies. Observed effects may not apply to other bone disorders such as OI and Paget's in which osteoblast phenotype and function may be distinct.

To conclude, in present study, we have identified OI related variants in type I collagen which suggests that the integrity of the triple helix is maintained by glycine residue if any substitution occurs by bulkier or charged amino acid residue, that leads to thermodynamically unfavourable protein structure and produces a kink in the triple helix. In addition to that triple helix, molecules are stabilized by chaperones and other cellular components. HSP47 appears to interact with triple helix indicating the involvement in transportation from ER to Golgi. Our findings suggest that HSP47 can be a future drug candidate. Owing to therapeutics, osteoclasts and osteoblasts are the targetes for the anabolic and catabolic drugs that help in improving bone mass and reduction of the fracture. Under the shadow concentration and duration of BPs-ZOl dose are important. With all these limitations we propose that sequel treatment rhPTH followed by ZOl will have as best anabolic effects.