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Review

Zebrafish; an emerging model organism for studying toxicity and biocompatibility of dental materials

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Abstract: Zebrafish (danio rerio) is a small, tropical freshwater teleost fish that belongs to the Cyprinidae family and lives in natural waters and rice fields in South Asia, North India, and Pakistan. Zebrafish has become a popular vertebrate model organism for biomedical research due to its numerous advantages such as their small size, short life cycle, accessibility in large numbers and inexpensive maintenance. In addition, fertilization happens externally in zebrafish and allows zebrafish to be manipulated directly. As another important advantage, the embryos are transparent thus the stages of development can be easily identified. Zebrafish can have multiple co-orthologs for human genes. In the 1930s, the zebrafish was first used as a model for developmental and embryological studies and in 1981, was introduced as a genetic model by Streisinger by force of developed genetic techniques in zebrafish such as cloning, mutagenesis and transgenesis. In the 1990s, various genetic manipulations were introduced. These improvements have contributed to the popularity of zebrafish. After that zebrafish was used in various research areas including genetics, biomedicine, neurobiology, toxicology, pharmacology as well as in human disease models. Zebrafish is also becoming a popular model organism in dental research. It is preferred in dental material toxicity studies and in research related to the genetic and molecular factors in tooth formation and craniofacial development. This review provides information on the use of zebrafish in dental research, focusing on tooth formation and dentition (pharyngeal dentition) of zebrafish and the dental research performed using zebrafish.

Key words: Zebrafish; Dental research; Model organism; Biocompatibility; Toxicity.

Introduction

Model organisms are used in biomedical and toxicological research to identify and investigate biological phenomena. Over time different organisms such as dogs, chimpanzees, pigs, rabbits, mice, rats, birds, *Drosophila* (fruit flies), *Caenorhabditis elegans, Arabidopsis*, E.coli, and zebrafish have been preferred according to their technical and practical advantages. In recent years, due to the completion of their genome sequences, the use of model organisms has increased in many areas of biomedical research (1). Having a have high homology with the human genome, rapid development, short life cycle, economically affordable maintenance, and being suitable for genetic applications are referred to as the main advantages of a model organism (2,3). Despite their long generation time and cost disadvantages, due to their similarity to the human genome and well-known biological structures, rodent models have been the most commonly used model organisms that provide the gold standard for biomedical research (2,3).

Recently zebrafish, a small teleost fish belonging the Cyprinidae family, has become a popular vertebrate model for biological studies (2). Zebrafish is a tropical freshwater fish that lives in natural waters and rice fields in the northern and northeastern India, Pakistan and South Asia (4). The length of an adult zebrafish is about 3-4 cm and there are 7-9 silver and blue stripes on its body (4,5). The optimal temperature for the incubation of zebrafish embryos and zebrafish breeding are 28.5 ° C (6,7).

In the 1930s, the zebrafish was first introduced as a model for developmental and embryological studies. The popularity of zebrafish increased between the 1970s–1980s as it became a new genetic model for forward genetic studies. In the 1990s, the zebrafish became the strongest model in developmental biology, owing to thousands of early developmental zebrafish mutants with genetic screens. Lately, the use of zebrafish for human disease has become increasingly popular (8).

Zebrafish reproduce rapidly: generally, each female spawns an average of 200 eggs in a week. It has a short generation time of 3-4 months. Maintenance of zebrafish research laboratories is easier than those working with rodents and other mammals (4). Additionally, large numbers of zebrafish can live in small tanks (9). In zebrafish, fertilization happens externally allowing it to be manipulated directly. External fertilization allows to accessing embryos directly to image the development. There is no placental barrier or maternal compartment. Therefore applying toxins or drugs to zebrafish by simple addition to the water is easier than other models (9, 10). Zebrafish embryos can ingest the diluted compounds from water through their skin and gills. For zebrafish, from 7 days post fertilization (dpf) to adulthood, drugs can be applied orally (10). Many human

and disease gene similarities are found in the genome of zebrafish. 76% of human genes have orthologues in zebrafish genes making zebrafish a preferred model in genetic studies (11). Moreover zebrafish embryos are transparent and the stages of its development can be easily identified and their functional movements, such as the heart beating are observable (1). Transparent and externally developing embryos are practicable models for genome-editing strategy. Zebrafish embryos develop rapidly, gastrulation completes in 10 hours post-fertilization (hpf), the heartbeat starts in 24 hours and, in 5 dpf most of the organs become functional (1,13). Embryogenesis is completed within 5 dpf. At 2-3 dpf larvae are inactive, at 4 dpf young zebrafish start swimming (13). Between 12 and 24 dpf zebrafish are referred to as young juveniles and from over 24 dpf they are considered as older juveniles. Zebrafish are considered adults when they gain sexual maturity in approximately three months (1,2,13)

On the other hand, zebrafish does not have some of the mammalian organs, such as heart septation, synovial joints, cancellous bone, lung, prostate, and mammary gland making it an unsuitable model to investigate the defects of these organs (14,15). Also there are differences in organ / body sizes between mammalians and zebrafish (14). Additionally, zebrafish genome has many gene duplications and the body temperature of the zebrafish is lower compared to the mammals (15). In contrast to mice and humans, zebrafish is cold-blooded, the body temperature of zebrafish depends on the ambient temperature, which is a limiting factor in some specific metabolic pathway studies. Therefore researchers using zebrafish as a model organism should take these fundamental differences between zebrafish and human into account and should be careful in interpreting the findings (14,15).

Zebrafish is commonly used as a model organism in different areas of biomedicine including neuroscience, cancer, toxicology, and pharmacology (16-21). In recent years, zebrafish has also been used as a model organism in dental research. This review provides information on the use of zebrafish in dental research focusing on pharyngeal dentition and the performed studies using zebrafish.

Development of zebrafish pharyngeal dentition

The zebrafish is a polyphyodont vertebrate, replacing teeth throughout its life like the other non-mammalian vertebrates. As with other cyprinids, zebrafish has no teeth in the oral cavity but has pharyngeal teeth that form from the fifth ceratobranchials (the ventral component of fifth branchial arch) also called the pharyngeal jaw (22). These components develop as a cartilage at 2 dpf when there is intense occurrence of chondroblasts. At 3 dpf, perichondral bone bundles the cartilage and at 4 dpf membranous apolamellae comes out from this bone to create the main shape of ceratobranchial (23).

The dentition on pharyngeal arches of zebrafish was shown by Cubbage & Mabee and Schilling et al. in previous studies (24,25) and pharyngeal teeth formation had been detailed in later studies (23). The zebrafish dentition is placed in three rostrocaudal tooth rows referred to as ventral (V), mediodorsal (MD) and dorsal (D). These rows consist of five, four and two teeth respectively (Figure 1a, 1b). These 11 teeth are named according to their position from rostral to caudal, in the ventral (1V–5V), mediodorsal (1MD–4MD) or dorsal (1D and 2D) tooth row (26). At 36 hpf there is no tooth germ appearance in the pharyngeal jaw. The first germs are visible in hatching (48 hpf or 2 dpf) and positioned posterior and medial to the intense fifth ceratobranchial cartilages (23).

Tooth formation progresses in five developmental stages: the initiation and morphogenesis, continuous morphogenesis, cytodifferentiation, attachment and, resorption and shedding (5). Tooth development starts in the ventral row from 2 dpf to 16 dpf, which is followed by the mesiodorsal (from 14 dpf to 24 dpf) and dorsal rows (from 24 dpf to 28 dpf) and the dentition completes in 26 days (5). At hatching, tooth 4V is understood to be the first germ to develop in dentition as it contains some matrix on its top (23). At 2 dpf it gets a bell shape (stage 2); the differentiation of 4V is completed at 3 dpf and at 4 dpf the tooth stabs the epithelium (23). Additionally, tooth 4V is the first attached (at 4 dpf) and shedded tooth (between 12 and 16 dpf) (5).

Although the teeth of zebrafish are different from human teeth, they have a similar structure. In both humans and zebrafish, tooth crowns are produced from dentin, which is covered with enameloid in fish and enamel in human as a protective layer. Dentine surrounds the pulp cavity. The dental pulp contains odontoblasts. Blood vessels and nerves are found only in the pulp cavity of the adult zebrafish. Larval pulp includes just a couple of odontoblast cells and the dentine has no tubules. On the other hand, as they have no permanent teeth no cementum is found in zebrafish (27) (Figure 2). The attachment

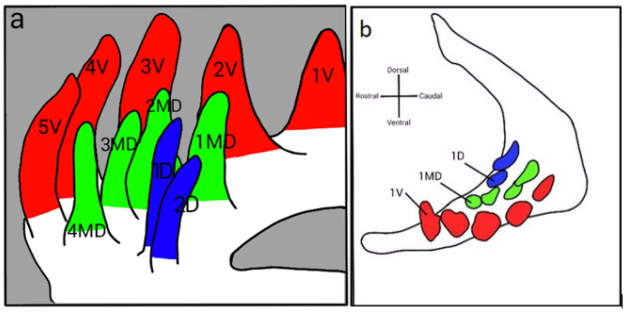

Figure 1. Figure 1: (a): Tooth positions in three rostrocaudal rows. **(b):** The dentition on the left pharyngeal jaw. Ventral teeth are shown as red circles, mesiodorsal are shown as green circles and dorsal teeth are shown as blue circles.

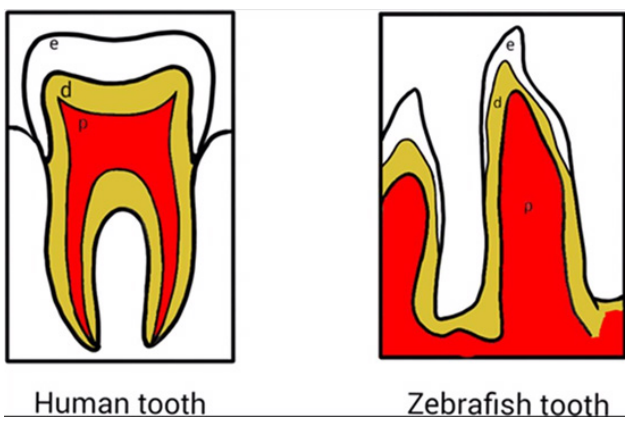

Figure 1. The shape of human and zebrafish tooth. e: enamel, d: dentine, p: pulp.

of the zebrafish teeth is maintained by a different bone tissue and this attachment known as ankylosis (28).

Vertebrate teeth are covered with a hypermineralized layer essentially of two varieties: enamel and enameloid. Enamel has only epithelial origin, while enameloid has both epithelial and mesenchymal origins (23). Enameloid, a dentine-like hypermineralized layer, coats the top of zebrafish tooth (first generation and juvenile – adult). Zebrafish enameloid includes collagenous components but the bulk of the matrix is non-collagenous, and the organic matrix mostly comprises tubular vesicles. The enameloid may include a wide range of carbohydrates. Some authors consider the collar enameloid as a true enamel, though others consider it differently (Peyer 1968). According to Smith et al. enameloid is the primitive tissue and the enamel originates phylogenetically from enameloid (29). The difference between enamel and enameloid occurs because of a heterochronic shift in the timing of epithelial cell differentiation relative to odontoblasts (23,29).The dentine of the zebrafish tooth is generated by mesenchymal cells, odontoblasts, and includes carbonated apatite crystals, type I collagen fibers and other proteins (28).

In larval and adult zebrafish, jaws and teeth differ in macroscopic size, therefore odontogenic alterations are expected. The first-generation teeth are smaller than the replacement teeth. In replacement teeth the enameloid matrix develops before the dentine formation. The other difference is the position of the initiation. The first-generation teeth develop severally from pharyngeal epithelium whereas the replacement teeth develops from an epithelial invagination which originates in accordance with the predecessor tooth (25-28). Each tooth has a neck and a crown part. The crown has two cusps- a major cusp on the top and a minor cusp on the ventral side; therefore, it is bilaterally asymmetrical. Tooth size and cusp depth change according to tooth position. Tooth 1V is the shortest, 3V is the tallest one. Tooth position also affects the neck-crown angle and the curvature of the tooth. Cusp depth increases from tooth 1V to 3V then decreases towards 5V. Both of these measurements increase from tooth 1V to 5V (13). There is no notable alteration in the tooth positions of the left and right pharyngeal jaws (26). From transversal cut, teeth appear as semicircular or elliptical shapes. Their bases contact each other closely (5).

In young juveniles, the functional lifetime of a tooth is approximately 8 days and at the end, the tooth is shed in consequence of a resorption of both the inner and the outer tooth surface (22). The dental organ of the replacement tooth develops near to the attachment site of the functional tooth. The position of the successor tooth is the same rostrocaudal direction, but a little ventral to the predecessor tooth that replaces it. The osteoclastic resorption of the functional tooth begins when the replacement tooth grows sufficiently (22). The order of tooth replacement of two American and one Japanese cyprinid fish has been formulated as 5V-3V-1V-4V-2V by Evans and Deubler and Nakajima (30, 31). On the other hand, Van der Heyden et al reported zebrafish dentition order as 5V-2V-3V-1V-4V (26).

Teeth develop in transversal rows and the new rows develop continuously behind the old ones. Teeth on the same transversal row are enumerated with the same

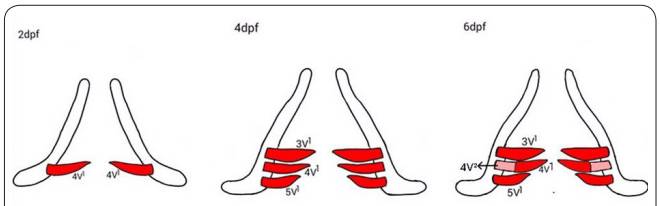

Figure 3. The first positioned tooth is 4V1 at 2 dpf; 3V1 and 5V1 follow 4V1 at 4-6 dpf.

number. As an exceptional row to this order, the row labeled as 2V arises in front of the first row (23). The primary teeth are positioned at 4V, 3V, 5V, and 2V becomes functional in two to four days, while the tooth positioned at 1V becomes functional in eight days (Figure 3). Most of the replacement teeth develop in 8 days. Therefore the functional period of the attached teeth differs between eight and twelve days (5).

Molecular control of tooth formation

Mouse and zebrafish models have been used to investigate the genetics of tooth development (32). In this part the studies about tooth initiation and replacement, and craniofacial development in zebrafish are explained.

N-cadherin is involved in many developmental processes and Verstraeten et al. evaluated the requirement of N-cadherine for cytodifferentiation in zebrafish. By using immunohistochemical methods they reported that N-cadherine is not found during the primary and replacement teeth' initiation and morphogenesis stages in zebrafish. On the other hand, during the differentiation of inner dental epithelium and the dental papilla cells N-cadherin is up-regulated. Moreover in the N-cadherin-deficient zebrafish, the development of the first tooth ended at the early cytodifferentiation phase and the development of the other first-generation teeth was totally repressed (33).

Wise and Stock investigated the necessity of bone morphogenetic protein (BMP) for zebrafish tooth development. BMP signaling is necessary in almost all stages of tooth development. They evaluated bmp2b and bmp4 expressions that are expressed in zebrafish teeth. They inhibited the function of these signals by using morpholino antisense oligonucleotides and reported that bmp2b and bmp4 elimination did not affect the formation of mature teeth (34).

Jackman et al. investigated the hedgehog signaling requirement in zebrafish tooth development. They reported that knockdowning a hedgehog ligand shha stops the mature tooth formation in every stage. In contrast, the increment of shha signaling does not affect tooth development (35).

Verstraeten et al. investigated the function of E-cadherin, an epithelial adhesion molecule, during the development of primary dentition and replacement dentition. By using in situ hybridization and whole mouth immunostaining they found that E-cadherin is situated in every layer of the enameloid during all development stages but there were insignificant differences between the first dentition and the replacement teeth (36).

Molecular aspects of modelling craniofacial diseases in zebrafish

Zebrafish have been suggested as a good model for

craniofacial development as most of the embryonic, anatomic, and genetic features of craniofacial development are conserved between zebrafish and mammals. Genome-wide association studies (GWASs) using zebrafish to discover the candidate genetic loci for craniofacial diseases using gene editing tools such as Gateway Tol2 and CRISPR-Cas9 have been greatly beneficial (37).

Neues et al. used zebrafish as a model of biomineralization in vertebrates. They used synchrotron radiation microcomputer tomography (SRlCT), scanning electron microscopy, polarized light microscopy, and energy dispersive X-ray analysis to observe biomineralization in teeth and bones. It was found that bones develop either by direct ossification or chondroidal ossification in zebrafish, similarly to human bones (38).

Smith et al. aimed to find out if Isthmin1 (ISM1) gene deletion contributed to the cleft lip and/or palate (CL/P) pathogenesis using zebrafish. Their results showed that ism1 is important in face and jaw growth and deletion of ism1 could lead to CL/P (39).

Neuhauss et al. specified 48 mutations in 34 genetic loci that result in craniofacial abnormalities by using zebrafish embryos. Phenotypic and genetic analyses showed that the specified mutations affected three different features of craniofacial development. In the first group, the mutations caused abnormalities in the whole form of the craniofacial skeleton, proving that the genes related to the identification of the rhombencephalon, neural crest, and pharyngeal endoderm were affected. In the second group, differentiation and morphogenesis of cartilage were affected. In the last group of mutations, abnormal arrangement was observed, revealing the significant tissue-tissue interactions in jaw growth (40).

In zebrafish embryo, seven arches are derived from the neural crest cells and Schilling et al. described 109 arch mutants focusing on the posterior pharyngeal arches and suggested that neural crest cells are specified in adjacent head segment groups by sets of genes that function in common genetic pathways in different types of tissues (41). Piotrowski et al. showed the phenotypic description and complementation analysis results of mutants in classes as the mandibular and hyoid arches defects and cartilage differentiation and growth defects (42).

The cranial neural crest (CNC) not only contributes to the peripheral nervous system but also contributes to the ectomesenchymal precursors in the head skeleton. Cox et al. showed that histone H3.3 replacement is essential during early cranial neural crest development. They also specified that h3f3a mutation disturbs the CNC-originated head skeleton and a subset of pigment cells (43).

Ignatius et al. (2013) used zebrafish hdac1 mutants to research the necessity of hdac1 in neural crest-derived craniofacial and peripheral neuron development. In hdac1b382 mutants, they observed defects in craniofacial cartilage development. Fewer hoxb3a, dlx2, and dlx3-expressing posterior branchial arch progenitor cells were defined and many of them resulted in apoptosis. They concluded that zebrafish hdac1 has important functions in neural crest-derived craniofacial development as well as peripheral neuron development (44).

Zebrafish as a model organism for studying biocompatibility of dental materials and molecular end points

Before the approval of a dental material to be used clinically testing in laboratory animals for its systemic and cytotoxic properties is manditory. Following toxic damage different cytotoxicity testings may be applied including lysosomal acid phosphatase, cytoplasmic lactate dehydrogenase and succinate dehydrogenase enzyme activities. Membrane integrity assay can also be measured as a biocompatibility assay. This assay measures the ability of cells to prevent impermeable extracellular molecules through colorimetric or fluorescent methods (45).

In recent years the zebrafish has become an emerging model organism in dental research. In this section, the studies performed using zebrafish and zebrafish embryo in researches about toxicity of the materials related to dentistry are outlined.

Fluoride is known to be an effective agent to prevent the formation of dental caries. On the other hand, excessive fluoride leads to fluorosis through an uncertain mechanism. The teeth of zebrafish contain a hard enameloid surface rather than true enamel. To find out if zebrafish could be a suitable model organism for dental fluorosis research Bartlett et al. exposed zebrafish to different concentrations of NaF for 8 weeks. In this study, pits and roughness, as well as increased organic components, were found in fluoride-treated teeth by using scanning electron microscopy and compositional analysis. They also reported that reduction of Alk8, a signaling molecule related with apoptosis, may affect fluorosis progression (46).

In another study, Zhang et al. used zebrafish larva to investigate the characteristics of dental fluorosis in primary teeth. They exposed zebrafish embryos to different fluoride concentrations (19 ppm, 38 ppm, and 76 ppm) for five days. They observed dose-induced fluorosis malformations. In analysis, the teeth cusps that were exposed to 19 ppm and 38 ppm fluoride were marked 1/3 from the top to bottom with the alizarin red and alcain blue, while in the control group, teeth cusps were marked totally red. The teeth that were exposed to 75 ppm fluoride were marked spotty with the alizarin red and alcain blue. H&E staining revealed that a cystic-like change occurred in the groups exposed to 38 ppm and 76 ppm. Also, dose-dependent changes were observed in zebrafish enameloid by using scanning electron microscope (SEM) technique (47).

The toxicity of zirconium oxide nanoparticles (ZrO_2NPs), that have been commonly found in biomedical applications such as dental implants were investigated on zebrafish model. Karthiga et al treated zebrafish embryos with different concentrations of nanoparticules during 24–96 hpf and observed the effects in embryonic stages with various analytical techniques. The dose of 0.5–1 µg/ml of ZrO_2NPs caused incitation to developmental acute toxicity, death, malformation and postpone hatching. At 1 mg/ml of ZrO_2NPs treatment, they observed the mortality of unhatched embryos as a common phenotype. According to their results, they suggested that lower concentrations of ZrO_2NPs nanoparticles are more toxic to zebrafish embryos (48).

Metal alloys are common used materials in den-

tal applications. Zhaoa et al. investigated the toxicity of gold-palladium (Au-Pd), silver palladium (Ag-Pd), Nickel chromium (Ni-Cr), cobalt-chromium (Co-Cr) and titanium (Ti) as porcelain-fused-to-metal crowns in zebrafish embryos. They put each of these alloys in artificial saliva for 1, 4, and 7 weeks. Zebrafish embryos treated with each of these solutions. Toxicity was measured according to mortality, spontaneous movement, heart rate, hatchability, malformation, and swimming behavior. They found that the most toxic alloy was Ni-Cr, followed by Co-Cr and Ag-Pd . In the same study, the biocompatible alloys were indicated to be Ti and Au-Pd (49).

Bioceramics are widely used materials in various dental clinical applications such as pulp capping agents, root-end fillings, perforation repair materials. Makkar et al. used embryonic zebrafish to evaluate the biocompatibility of two popular bioceramics: mineral trioxide aggregate (MTA) and biodentine. Zebrafish embryos were exposed to different concentrations of these dental materials and apoptosis and ROS induction were assessed to analyze toxicity. They found morphological malformations, decreased survivability rates with the increasing concentration of materials. Biodentine was found to be more biocompatible than MTA (50).

Bisphenol A-glycidyl methacrylate (Bis-GMA) is a dental filling composite monomer and Kramer et al. exposed zebrafish embryos to Bis-GMA at 12 hpf and investigated the effects on craniofacial development. Bis-GMA caused abstruse malformation in the cartilage of the jaw and obvious morphological defects depending on the concentration. 1 μM and 10 μM concentrations caused 30% and 45% mortality rates respectively. At a concentration of 10 nM, the macro defects including craniofacial abnormalities were found (51).

Methacrylate (MA) is used in biomedical devices, restorative dental composites and in bone cement. Altayib et al investigated the effects of MA exposure on developing zebrafish embryos and reported that MA exposure had no significant effect on the nitric oxide levels of the embryos. However, they reported some developmental defects and pericardial edema in some of the embryos exposed to MA. They concluded that zebrafish embryos are useful models for the evaluation of dental MA toxicity (52).

Zebrafish does not have some dental structures including the periodontium. Nevertheless Widziolek et al. used zebrafish to investigate the role of *Porphyromonas gingivalis (P. gingivalis)* pathogenicity, the main pathogen that leads to severe periodontitis, in cardiovascular diseases. They showed that *P. gingivalis* can cross the vascular endothelium and diffuse into the surrounding tissues and cause pericardial oedemas and cardiac damage leading to mortality (53).

Conclusion

The high reproduction capacity, short generation time and easier maintenance compared to rodents make zebrafish a useful model organism in biomedical research. The disadvantages of zebrafish as a model organism include the lack of some mammalian organs, such as periodontium, heart septation, synovial joints, cancellous bone, lung, prostate, and mammary gland. Researchers using zebrafish as a model organism should take into account these fundamental differences between zebrafish and human and should be careful in interpreting the findings. As a polyphyodont organism zebrafish dentition is replaced continuously throughout life and E-cadherin has important roles in the continuous tooth renewal. Nowadays, zebrafish have also found use in dental research, especially in biocompatibility of dental materials, craniofacial development and toxicological research. The popularity of zebrafish is expected to increase leading to the enhancement of both the amount and quality of dental research.

Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author's contribution

Gözde Ece Karaman wrote the review, Ebru Emekli-Alturfan and Serap Akyüz edited the paper.

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