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In vivo Molecular Functional Aging and Gradual Senescence with hTERT Gene as Antiaging Interventions in *Danio rerio*

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Authors' contributions

This work was carried out in collaboration among all authors. The work was carried out and designed by the First Author RK and the manuscript was prepared by him. All other authors have read and approved the final version of the manuscript.

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ABSTRACT

The study investigates the impact of resveratrol on zebrafish, a model organism, to understand the enzymatic responses and gene expression changes associated with aging. Zebrafish are significant in understanding human diseases and comparing them with zebrafish. The study reveals significant

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variations in muscle catalase, muscle super peroxide, and muscle dismutase, with a focus on the hTERT gene. The study also reveals senescence-associated activity in muscle and abundant telomerase activity during aging. The study suggests that telomerase activity is strongly correlated with telomere shortening and replicative senescence. The study also compares resveratrol-treated zebrafish to the control group, revealing that intermediate concentrations of resveratrol show variations at the muscle level and prevent muscle damage. The results suggest that resveratrol treatment in zebrafish retains dye and has a positive effect on muscular tissue. These findings provide comprehensive knowledge in antiaging research and could potentially aid future research.

Keywords: Zebrafish; aging; enzymatic activity; gene expression; telomerase; telomere.

1. INTRODUCTION

Nowadays, antiaging research is an exciting factor for a healthy lifestyle. Most of the inventions focused on ageing-related diseases, revealing the anti-aging therapeutics in humans. The process of aging, a non-genetic intervention, plays a crucial role in increasing the risk of cognitive decline related to neurodegenerative disorders. This emphasizes the importance of comprehending aging as a means to prolong both lifespan and health span [1]. Telomerase plays an emerging role in antiaging. Mutations in telomerase in humans have led to the emergence of syndromes characterized by premature aging. Werner syndrome and Hutchinson-Gilford syndrome are two extremely rare genetic illnesses that are included in the group of premature ageing syndromes. Skin alterations that point to early aging in both diseases include skin wrinkling and thinning and loss of elasticity. Telomerase is necessary during zebrafish's lifespan, and telomerase deficiency leads to senescence responses [2].

In various laboratories, aging models are utilized to comprehend the fundamental principles underlying the process of aging. While these models contribute to the investigation of basic principles, it is important to note that the relationship between lifespan and the aging phenotype is not universally consistent across diverse species [3]. Despite variations in aging characteristics and patterns among different species, zebrafish have been proposed as a valuable model for conducting anti-aging studies due to their resemblance to human aging [4].

Telomeres can lose their functionality as a result of significant shortening, and oxidative damage [5]. Telomeres, which are nucleoprotein structures located at the ends of eukaryotic chromosomes, play a crucial role in safeguarding against chromosome fusion and degradation. When telomeres become excessively short, they trigger a DNA damage response that ultimately results in the activation of a cellular cycle known as senescence [6].

Zebrafish exhibit abundant and continuous telomerase activity, particularly in adult somatic tissues, suggesting that their cells possess the ability for unlimited replication. While certain stress-related markers are upregulated and minor histological alterations are observed during the aging process of zebrafish, their senescence occurs gradually over time [7]. Aging occurs as a consequence of telomere shortening, wherein telomeric repeats are progressively lost with each replication event. In zebrafish, the mean length of telomeres decreases significantly from the young adult stage to the older adult stage, indicating a significant reduction in telomere length [8].

A deficiency of telomerase in zebrafish results in p53-dependent premature aging and reduced lifespan, which may indicate a similarity in telomere length dynamics between fish and humans [9]. Telomeres, found in the majority of organisms, consist of repetitive sequences that are responsible for maintaining their length through the action of the telomerase ribonucleoprotein [10]. Telomerase is comprised of two vital components: telomerase reverse transcriptase (TERT) and telomerase RNA. Telomerase RNA serves as the template for the reverse transcription process carried out by TERT, resulting in the synthesis of new telomere DNA [11].

The shortening of telomeres and instability of chromosomes occurred due to the loss of improper function of telomerase leading to aging [12]. The functional characterization of zebrafish is based on telomerase reverse transcriptase which is involved in the process of vertebrate development [13]. Telomerase plays a critical role in elongating telomeres, thereby protecting chromosome ends from recombination and

fusion. The absence of this enzyme leads to cellular DNA damage responses, regardless of any alterations in telomere integrity [14]. Telomerase is the catalytic component and it served as the template for DNA repeat synthesis [15]. Telomerase reverse transcriptase has characterized by functional motifs and domain [16]. Telomere integrity which is responded for DNA damage and TERT expression in different vertebrates may be associated with functions of stem cells and regenerative abilities [17]. The relationship among telomerase activity, telomere structure and telomere length is based on the TERT and activities of the cell [18]. The functional involvement of the TERT gene plays a critical role in telomerase in aging [19].

During the process of aging, the factors and mechanisms evaluated in early development may be considered as senescence [20].

2. MATERIALS AND METHODS

2.1 Maintenance of Zebrafish

Zebrafish (AB strains of Zebrafish were used for our investigation) were raised and kept in controlled laboratory conditions at a temperature of 28.5°C, following a 14-hour light and 10-hour dark cycle. Zebrafish are characterized and subjected to morphological criteria. Zebrafish are usually fed once a day and the frequency of resveratrol administration to fish's water has maintained to ensure constant exposure. The different concentrations of resveratrol can be administered to investigate the effects of zebra fish on various biological processes and specific health conditions [21,22].

2.2 Experimental Protocol

Zebrafish are categorized in to four groups including control based on the enzymes responsible for aging. Resveratrol is a potential anti-aging compound so that zebrafish are characterized based on the concentration of resveratrol. They are reared in water that maintains a temperature between 26°C and 28°C. Within the range of 6.8 to 7.8, the pH of the water was maintained. The quantities of dissolved oxygen were maintained at roughly 6–8 mg/L. The aging factors are evaluated in the muscle so that the enzymes muscle catalase, muscle superoxide dismutase and muscle lipid peroxidase are treated with zebrafish with 0.5, 1.0 and 1.5 µmole/mg of protein respectively. There are 120 fishes are involved in this study and each group has separated with 30 fishes based on the concentrations. Each group of fishes is treated with various concentrations and the results were observed. Zebrafish was treated and observed at the initial day, 30th day, 60th day and 90th day. It can be observed for 90 days.

2.3 Sample Preparation

The frozen muscles were crushed and sonicated on ice in a buffer containing 20 mM MOPS, 620 mM sucrose, and 1 mM EDTA at pH 7.2. The resulting mixture was subjected to centrifugation at 13,000 × g for 30 minutes at 4°C. The protein concentration in the obtained supernatant was determined using the Bradford assay (Bradford, 1976). To the extracted samples, cold catalase buffer (composed of 50 mM potassium phosphate monobasic and 50 mM sodium phosphate dibasic at pH 7.0) was added, and the reaction was initiated using H2O2. The absorbance at 240 nm was measured to calculate the value [23].

2.4 Gene Expression

From the treatment, periodically samples has been collected and sacrificed the model to collect muscle samples for gene expression studies. The current gene expression was performed with 90^{th} day samples.

2.5 Extraction of Total RNA

The RNA extraction process involved the use of TRIZOL, A 50 mg sample was thoroughly mixed with miliQ-grade water and centrifuged to remove impurities. Then, 700 µl of TRIZOL was added to the pellet for cell lysis. The resulting lysate was transferred to 1.5 ml tubes, and 200 µl of chloroform was added. After vigorous mixing, the mixture was centrifuged at 12000 rpm for 25 minutes at 4°C, separating the aqueous layer. The aqueous layer was carefully collected and transferred to a new tube. To precipitate the RNA, 700 µl of isopropanol was added to the aqueous layer, and the mixture was centrifuged at 12000 rpm for 20 minutes at 4°C. The resulting RNA pellet was washed with 70% ethanol and then air dried. Finally, the RNA pellet was dissolved in 30 µl of distilled autoclaved water and stored at -80°C until further use. The Labman UV Vis Spectrometer was employed to determine the quantity of RNA, while the quality was assessed by resolving the RNA in a 1% agarose gel with ethidium bromide.

2.6 DNase Treatment

During RNA preparation, DNase treatment was performed to eliminate any potential contamination. To carry out this step, a reaction volume of 20 µl was prepared, containing 1 unit of DNase. The mixture was then incubated at 37°C for a period of 30-45 minutes. After the incubation, the DNase was heat-inactivated by subjecting the reaction to a temperature of 85°C for 3 minutes. The treated RNA was then stored for subsequent processing.

2.7 Synthesis of cDNA

Approximately 1.5 µg of total RNA was transformed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit. The cDNA synthesis process involved incubating the mixture at 25°C for 10 minutes, followed by 37°C for 120 minutes. To denature the cDNA and RNA hybrid and inactivate the reverse transcriptase enzyme, the reaction was subjected to a temperature of 85°C for 2 minutes. The resulting cDNA was then utilized as a template for gene expression analysis.

2.8 Real-Time PCR

For the current study, all quantitative gene expression analyses were carried out by real time PCR using SYBR Green chemistry with the primer. These primers are retrieved based on the evidence from the literatures [24]. The primers are used for the analysis is zftrt-2750 as 5'-CGGTATGACGGCCTATCACT-3' and zftrt-2997 as 3'- AACGACGGGTTTCTGTTCAC- 5' and for protein forward 5'-GTGCCCATCTACGAGGGTTA -3'and reverse 3'- TCTCAGCTGTGGTGGTGAAG - 5'.

For each reaction mix the following composition was prepared to perform the analysis. The component volume 2X SYBR Green master-mix 5 µl, forward primer (5 µM) 0.5 µl, reverse primer (5 µM) 0.5 µl, cDNA template 1 µl and nuclease-free water 4 µl are mixed together and carry out the experiment. Each gene was assayed in duplicates for every sample and the reactions were run in 96-well plates sealed with clear sealing films. The following temperature program was used for amplification. The temperature time for initial denaturation is 94°C 5 min and 45 cycles of 94°C for 30 sec during cyclic denaturation and for annealing 60°C for 30 sec. For signal acquisition 72°C for 30 sec is used. Data analysis: Data was analysed using inbuilt software with the machine. Ct values were obtained for each well and normalized Ct values were calculated by subtracting the Ct values of internal control gene or reference gene from those of the target gene. Mean of these normalized Ct values were plotted. Moreover, fold change in expression was calculated inbuilt software by the ΔΔCt method. For fold change of gene expression in target as compared to that in control: ΔΔCt= ΔCt of sample- ΔCt of control.

2.9 Telomere Length Assay

At the end of the study period, one fish of each group was randomly chosen and sacrificed and subjected to telomere length assay. as per previously described [25].

2.10 Extraction of Genomic DNA from Muscle Sample (Kit Manufacture-QIAgen)

50mg of muscle sample of each fish was taken in a 1.5ml microcentrifuge tube and added 1ml of PBS was to the sample and centrifuged at 1800 xg for 5 minutes at room temperature (looked for the pallet if not, given one more cycle of centrifugation at 1800xg for 5 minutes in room temperature). Carefully remove the supernatant and resuspend the pallet in 180µl of PBS to homogenize the tissue with a handheld homogeniser. With the tissue suspension 20µl of Qiagen protease to the sample followed by 200µl of buffer AL to the sample and mixed by vortexing immediately. Incubated at 56° C for 10 minutes. After incubation spin down the tubes and added absolute alcohol and mix by vortexing. Transferred the content into a Qiamp spin column, placed in a 2ml collection tube, without touching/wetting the rim and centrifuged at 6000xg for 1 minute. Discarded the filtrate, placed the spin column in a clean collection tube and then added 500µl of buffer AW 1 to the sample, centrifuged at 6000xg for 1 minute. Discarded the filtrate and transferred the spin to column to a clean collection tube and added 500 µl of buffer AW 2. Centrifuged at full speed for 3 minutes. Discarded the collection tube after filtration placed the Qiamp spin column in a clean 1.5ml microcentrifuge tube to the column and added 100 µl of buffer AE. Incubated at room temperature for 1 to 2 minutes and centrifuged the tube for 1 minute at 8000rpm. The extracted DNA concentration was confirmed through measurement by NanoDrop 2000c spectrophotometer. The remaining

samples were Stored at -20° c Futures studies.

2.11 Telomere Length Estimation

Relative telomere length was expressed as 1/(Ct telomere/Ct Single Copy Gene) as described earlier. The protocol used was a modified version of the method described by Cawthon, briefly the isolated DNA vials were allowed to warm room temperature, centrifuged the vials at 1,500x g for 1 minute. For each sample Two qPCR reactions was prepared, one with telomere primer stock solution and one with Single copy Reaction primer stock solution for the reference genomic DNA sample. Sealed the qPCR reaction wells. Centrifuged the plates at 1,500x g for 15 seconds. By adding Fast Start Essential DNA Green master mix the qPCR program was Telomere length qPCR reactions were performed at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 54°C for 2 min. The c-fos gene was used as single copy gene as reference for the relative ratio.

2.12 Quantification Method: Comparative ΔΔCq (Quantification Cycle Value) Method

For telomere (TEL), ΔCt (TEL) is the quantification cycle number difference of TEL between the target and the reference genomic DNA samples. It was calculated by ΔCt (TEL) = Ct (TEL, target sample) - Ct (TEL, reference sample). For single copy reference (SCR), ΔCt (SCR) is the quantification cycle number difference of SCR between the target and the reference genomic DNA samples and it was calculated by ΔCt (SCR) = Ct (SCR, target sample) - Ct (SCR, reference sample) ΔΔCt = ΔCt (TEL) - ΔCt (SCR). Relative telomere length of the target sample to the reference sample (fold) = $2^{-\Delta\Delta Ct}$. Total telomere length of the target sample = Reference sample telomere length x $2^{-\Delta\Delta Ct.}$

2.13 Histopathological Analysis

2.13.1 Zebrafish sample collection

The zebrafish can be randomly chosen from each group of the experiment includes control, group 1, 2 and 3. The control group is normal zebrafish whereas the fish taken from the other groups are resveratrol treated zebrafish with various concentrations.

2.13.2 Sample preparation

Zebrafish muscle samples are typically obtained by dissecting specific muscles from the fish. After that, the samples are placed in a formaldehyde solution to maintain the cellular structure and prevent deterioration. The samples were fixed for 24 hours at room temperature in neutral buffered 4% paraformaldehyde. The samples were subsequently dehydrated, infiltrated, embedded in paraffin, cut using a pathologic microtome into 4-m-thick slices, mounted on glass slides, and heated at 60°C. Then, tissue sections were stained with hematoxylin and eosin and examined under an optical microscope at 400x magnification [26].

2.13.3 Embedding

The infiltrated tissues are embedded in blocks of solidified paraffin wax. The infiltrated tissue is placed into a mold containing more liquid paraffin wax. The mold is then cooled, causing the paraffin to solidify and form a block around the tissue. This block contains the tissue specimen embedded within it. Once the paraffin block is solidified, excess paraffin is trimmed away from the sides of the block to expose the tissue surface.

2.13.4 Sectioning

The paraffin block containing the embedded tissue is placed on a microtome, which is a precision cutting instrument. Thin sections of the tissue are cut from the block using a sharp blade usually around 5-10 micrometers thick from the embedded tissue blocks. Sections are floated in a water bath and collected onto glass microscope slides.

2.13.5 Staining

Sections are deparaffinized and rehydrated to remove wax and rehydrate tissues. These sections are then stained using histological stains, such as Hematoxylin and Eosin to highlight cellular components and structures. The specialized stains may be used to target specific elements of interest, such as muscle fibers, connective tissues and blood vessels.

2.14 Microscopic Analysis

Light microscopy is used to analyze the stained sections in order to see the cellular structure, muscle fibers, nuclei, and any abnormalities. It

helps to observe the muscle damage inflammation, fibrosis or other pathological changes.

2.15 Statistical Analysis

The obtained data were analyzed by using the one-way ANOVA – SPSS Statistics software**.** The continuous variables of the normal distribution were expressed as mean ± standard deviation, and the results were expressed in standard deviation, standard Error and Average.

3. RESULTS

3.1 Morphological Observations for Resveratrol Treated Zebrafish

Zebrafish are raised and maintained at specific temperature and calculate the weight and length of the zebrafish. Fig. 1(a): Senescence associated zebrafish was observed at initial day of our analysis in Control, Group I, Group II and Group III Fig. 1(b): Resveratrol treated zebrafish observed at 30th day our analysis in Control, Group I, Group II and Group III. Fig. 1(c) Resveratrol-treated zebrafish were detected on the 60th day of observation Fig. 1(d) Resveratrol-treated zebrafish examined on 90th day of observation in Control, Group I, Group II and Group III.

3.2 Enzymatic Assay

At the initial day of analysis, the value for protein in control shows as 210 ug/ml and the value for groups 1, 2 and 3 as 218,180 and 168 respectively. At the catalase, the highest value was designated in groups 2 and 3 as 29.7 and 28.6 respectively. Superoxide dismutase has 5.8 in groups 2 and 3 and lipid peroxidase has 1.3 in group 1(Table 1).

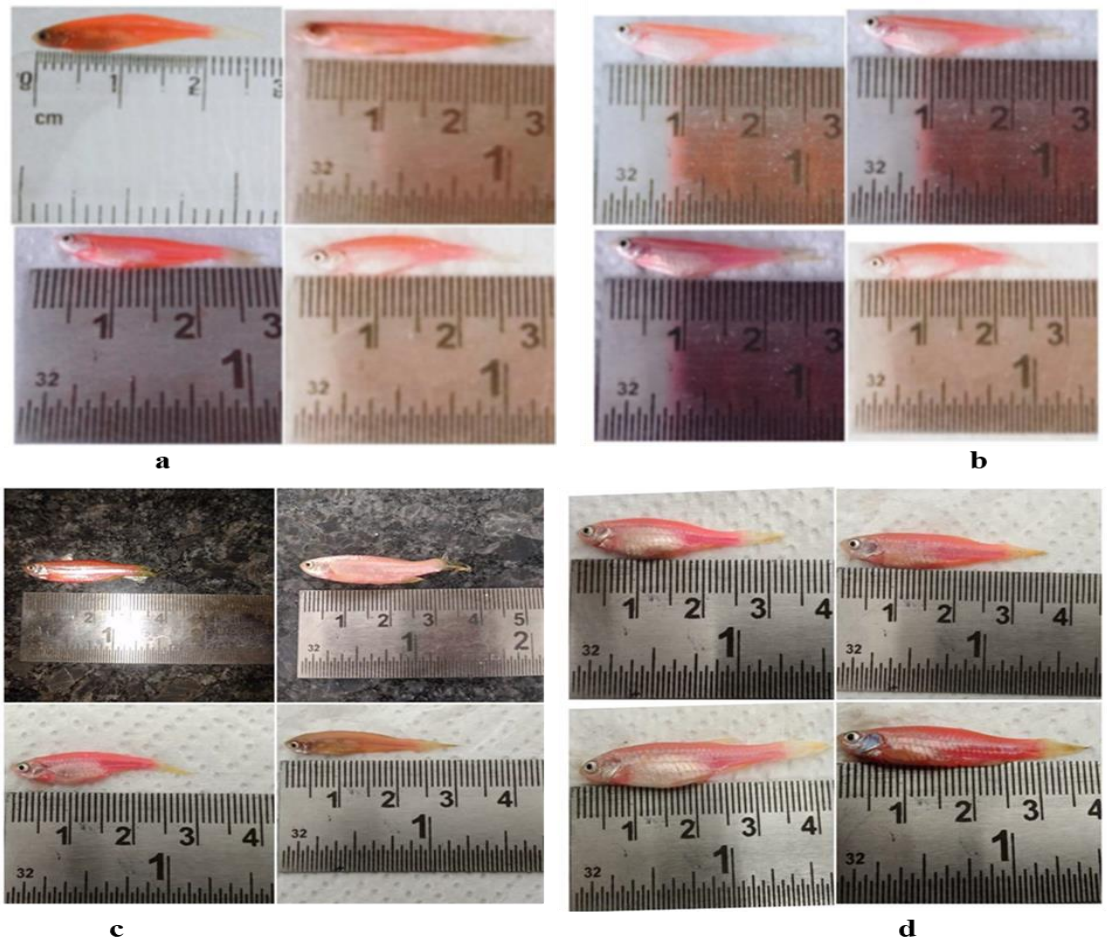

Fig. 1. Morphological observations for resveratrol treated zebrafish: (a): 0th day of observation for control and other groups, (b): 30th day of observation for control and other groups, (c): 60th day of observation for control and other groups, (d): 90th day of observation for control and other groups

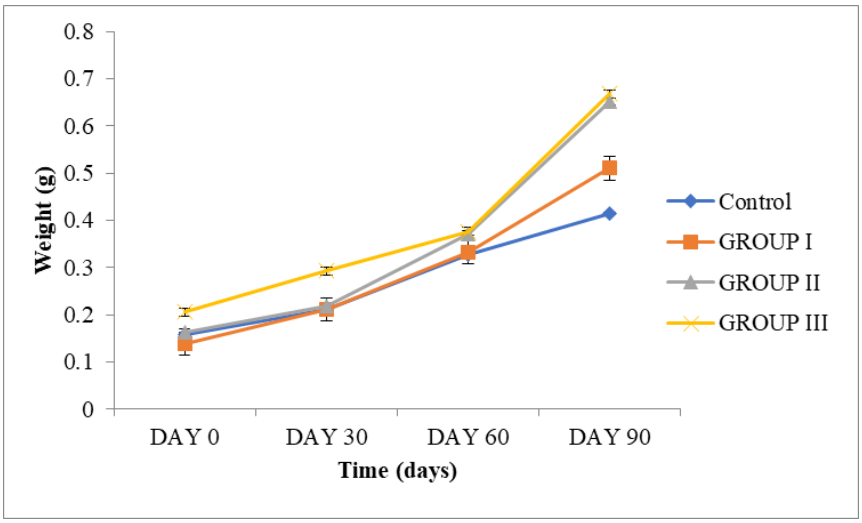

Fig. 2 Biomass for resveratrol treated zebrafish

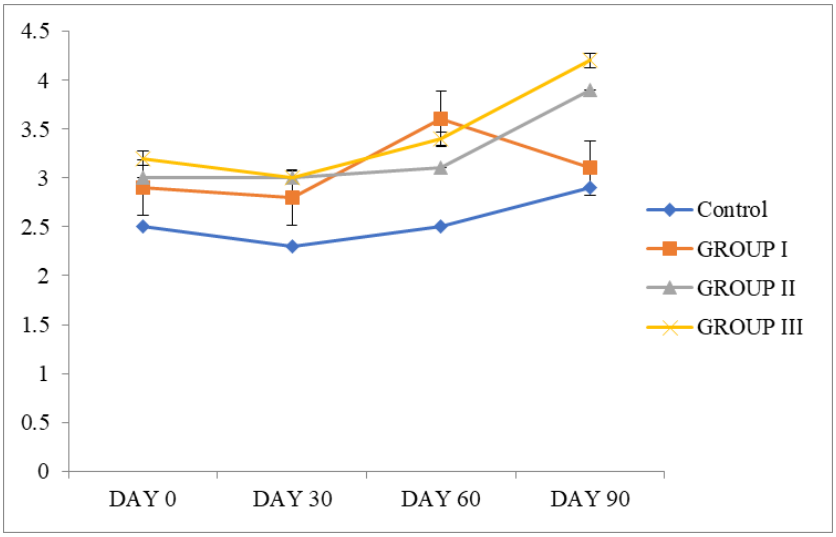

Fig. 3 Length for resveratrol treated zebrafish

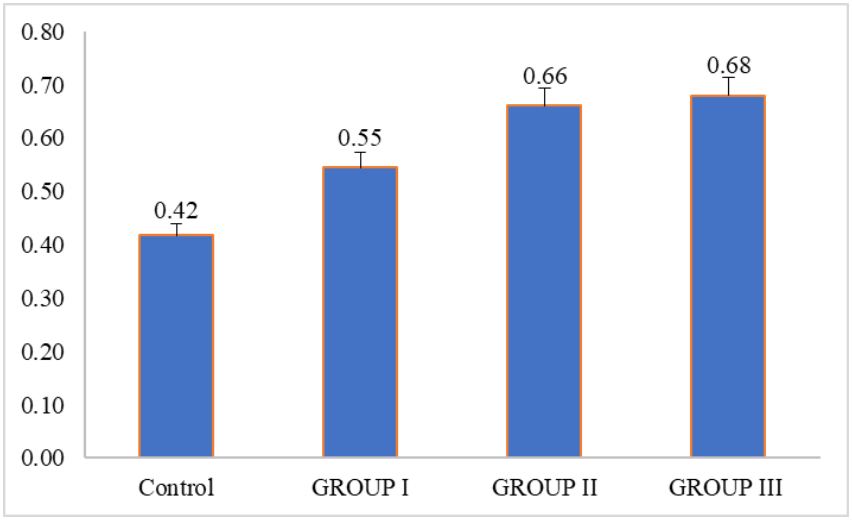

Fig. 4. Bar diagram of biomass for resveratrol treated zebrafish

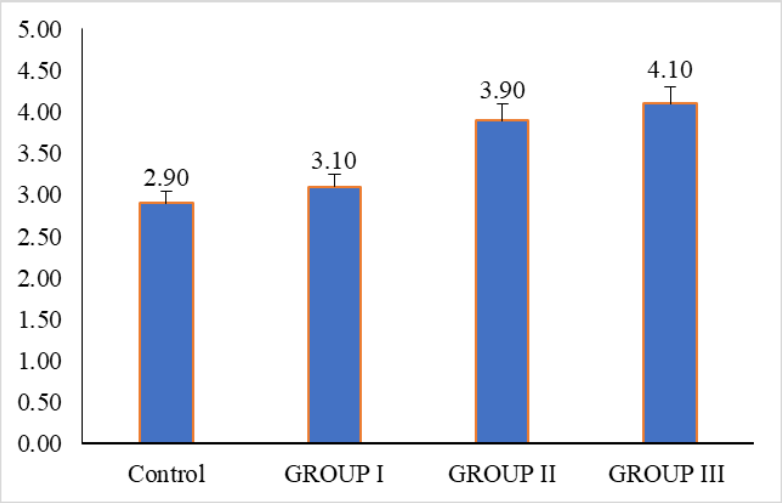

Fig. 5. Bar diagram of length for resveratrol treated zebrafish

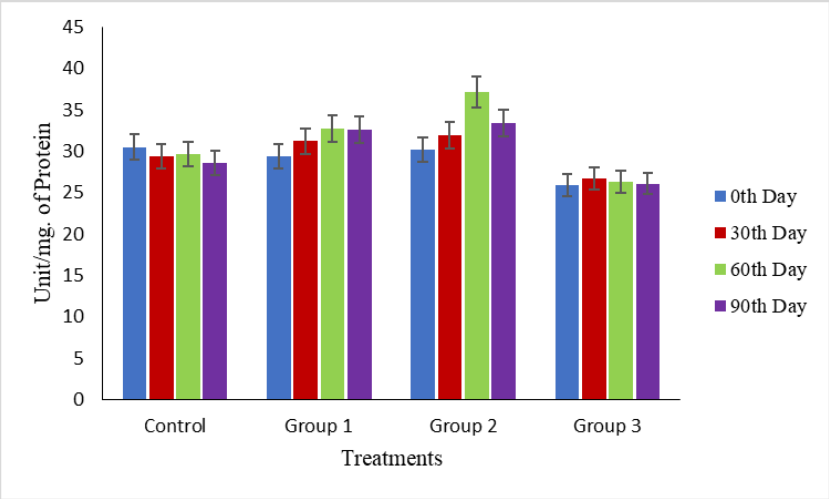

Fig. 6. Observations from muscle catalase at various stages of treatment

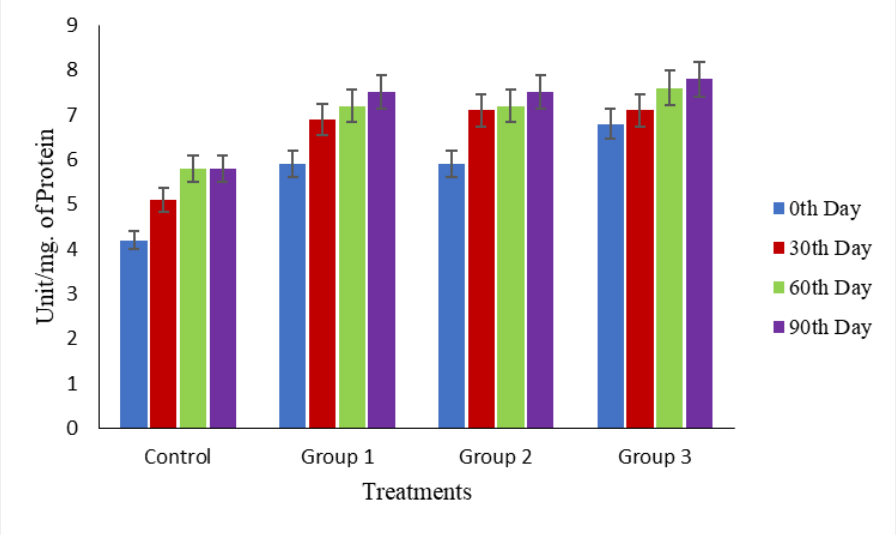

Fig. 7. Observations from muscle superoxide dismutase at various stages of treatment

Table 1. Enzymatic assay at 0th day in zebrafish

Treatment	Protein (ug/ml)	Catalase (Unit/mg. of protein)	Superoxide dismutase (SOD) (Unit/mg. of protein)	Lipid PerOxidase (µmole/mg. of protein)
Control	210	30.5	4.2	1.2
Group 1	218	29.4	5.1	1.3
Group 2	180	29.7	5.8	1.1
Group 3	168	28.6	5.8	1

Table 2. Enzymatic assay on the 30th day in zebrafish

Treatment	Protein (ug/ml)	Catalase (Unit/mg. of protein)	Superoxide dismutase (SOD) (Unit/mg. of protein)	Lipid PerOxidase (µmole/mg. of protein)
Control	224	29.4	5.9	1.2
Group 1	264	31.2	6.9	1.3
Group 2	226	32.7	7.2	1.4
Group 3	216	32.6	7.5	1.4

Table 3. Enzymatic assay at 60th day in zebrafish

Treatment	Protein (ug/ml)	Catalase (Unit/mg. of protein)	Superoxide dismutase (SOD) (Unit/mg. of protein)	Lipid PerOxidase (µmole/ mg. of protein)
Control	198	30.2	5.9	1.2
Group 1	224	31.9	7.1	1.4
Group 2	231	37.2	7.2	1.5
Group 3	195	33.4	7.5	1.5

Table 4. Enzymatic assay at 90th day in zebrafish

Treatment	Protein (ug/ml)	Catalase (Unit/mg. of protein)	Superoxide dismutase (SOD) (Unit/mg. of protein)	Lipid PerOxidase (µmole/mg. of protein)
Control	245	25.9	6.8	1.4
Group 1	264	26.7	7.1	1.3
Group 2	152	26.3	7.6	1.6
Group 3	206	26.1	7.8	1.4

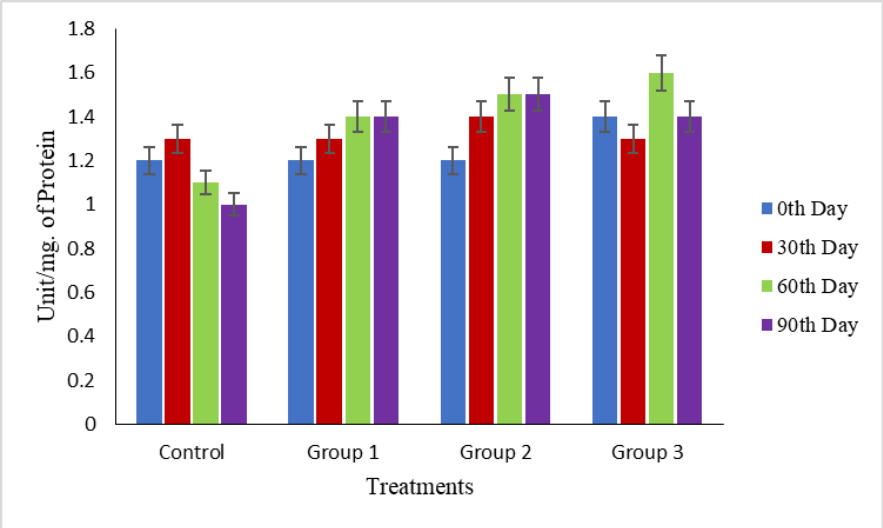

Fig. 8. Observations from muscle lipid peroxidase at various stages of treatment

On the 30th day, the results were observed based on the enzymatic activity and it indicates 32.7 and 32.6 Units/mg. of protein in group 2 and 3 respectively. The Superoxide dismutase has 7.5 units/mg of protein in group 3 and lipid peroxidase has 1.4 in group 2 and 3. In the increased concentration, zebrafish enzymatic activity shows the highest value in group 2 and 3 (Table 2).

The enzymatic assay was performed and the results were assessed at the 60th day in zebrafish and it expresses 37.2 for catalase activity in group 2 whereas 7.5 for superoxide dismutase in group 3 and 1.5 for lipid peroxidase in group 2 and 3. In the group 2 and 3, it indicates the enhancing activity of enzymes in zebrafish (Table 3).

The results for the zebrafish were assessed by the enzymatic assay at 90th day of observation, it indicates that 26.7 unit/mg of protein in group 1 for catalase while 7.8 was observed at group 3 for superoxide dismutase and 1.6 was determined at group 2 for lipid peroxidase. In the highest concentration, the mode values are observed for enzymatic assay (Table 4).

3.3 RNA Quality Assessment

RNA quality assessment in zebrafish indicates evidence for gene expression. Fold variation was calculated based on 2^- ΔΔCt. The fold variations were evaluated by RNA quality checking for all the groups (Fig. 9).

The fold variations can obviously observe in group 2 when compare with control group. The fluctuation in the fold variations was observed in control and other groups. In the lowest concentration (0.5µ ml), it indicates that the lowest variation while in the moderate concentration (1.0µ ml), the highest fold variation was observed. Even in the highest concentration (1.5µ ml), the variation is very less (Fig. 10).

3.4 Telomere Length Assay

Fig. 11 displays the amplification plot of hTERT gene expression and it indicates that the magnitude of the normalized fluorescence signal for analysis. Fig. 12 represents that the amplification plot for telomere length assay.

Table 5 represents that the quantifications of telomeres length estimation using delta ct method. Telomere length per diploid cell and telomere length at each chromosome end was observed as 751.79 and 9.95 respectively in group 3.

3.5 Histopathological Analysis

Fig. 13.a shows the histological findings in the zebrafish muscle at a 40 X magnification. The highly structured and compact tissue was visible in the control organism. Myofilaments are arranged regularly and compactly, with peripheral nuclei and a thin layer of connective tissue separating the fiber bundles. Fig. 13.b indicates that histopathological observations in muscle of resveratrol treated zebrafish exposed to 0.5 µml concentration at 40 X magnifications. The treated organism shows the alteration in the staining affinity leads to modifications in the connective tissues.

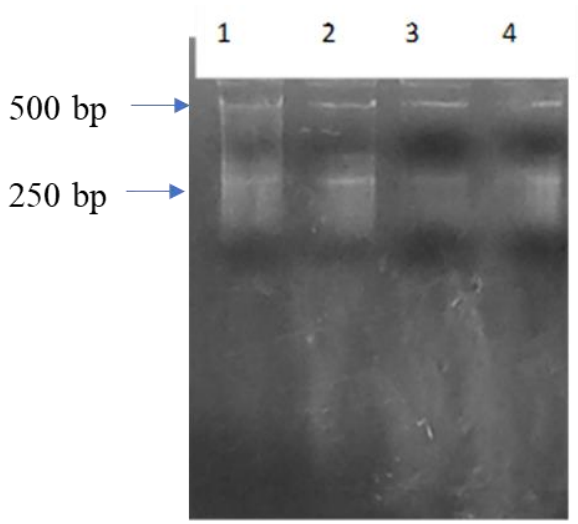

Fig. 9. RNA Quality checking AGE picture

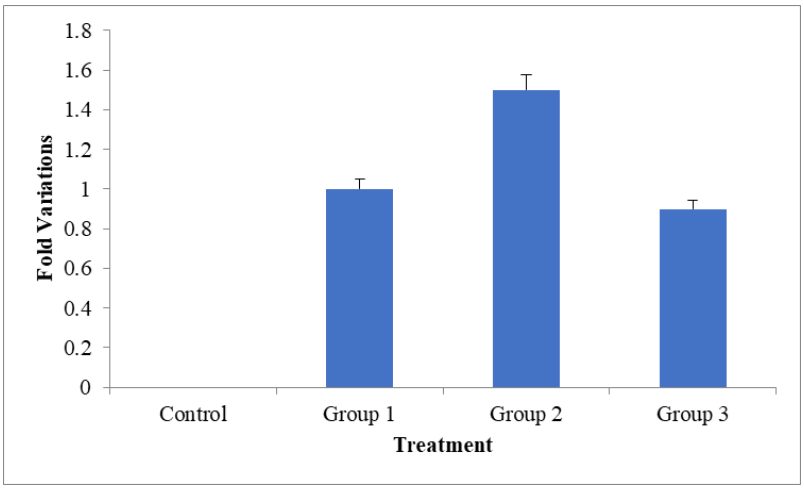

Fig. 10. Relative fold variation of zftrt genes different treatment groups compare with control group

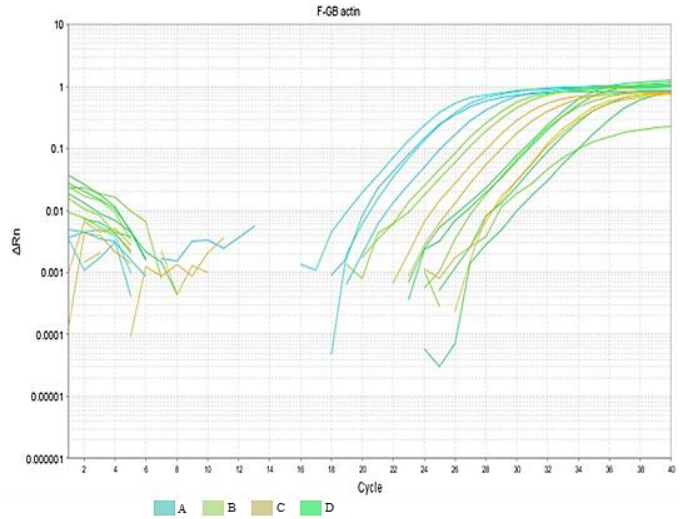

Fig. 11. Amplification plot of gene expression studies (A-Control; B-Group 1, C-Group 2; D-Group 3)

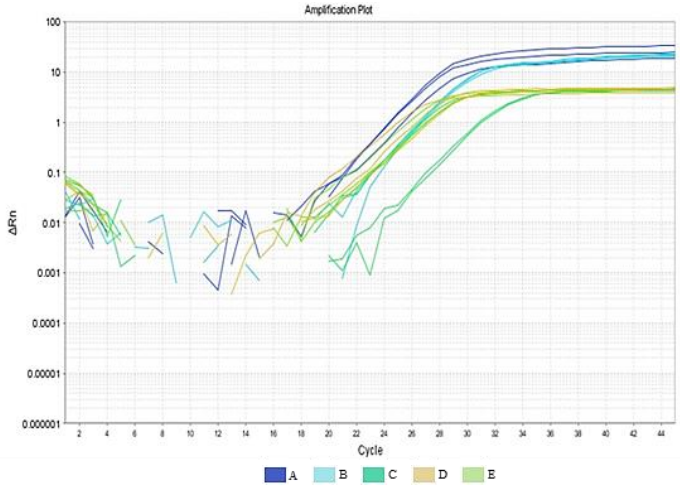

Fig. 12. Amplification plot of telomere length assay (A-Control; B-Group 1, C-Group 2; D-Group 3, E-Group 4)

Table 5. Quantifications of telomeres length estimation

	Telomere		SRC		ΔΔ Ct	2^-ΔΔ Ct	Telomere Length per diploid cell	Telomere length /each chromosome end	Telomere length/each chromosome end X 10^3 Kb
Sample ID	**CT**	**ΔCT-telomere**	**CT**	**ΔCT-SRC**					
Reference	13.8283	0	10.8158	0	0	1	695	7.55	
Group 1	13.652	-0.176	10.6972	-0.1186	-0.0577	1.04	723.36	7.86	0.00786
Group 2	13.2314	-0.597	10.2065	-0.6093	0.0124	0.99	689.05	7.49	0.00749
Group 3	12.9219	-1.192	10.0214	-0.7944	-0.398	1.32	751.79	9.95	0.00995
Group 4	13.433	-0.395	10.0141	-0.8017	0.4064	0.75	524.38	5.70	0.00570

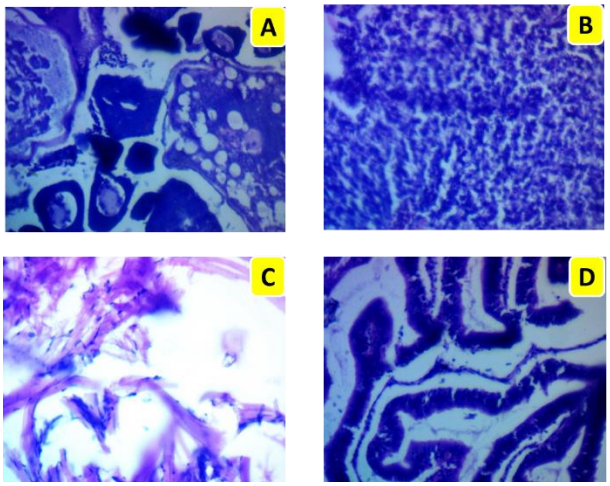

Fig. 13. Histopathological observations of Zebrafish, A) Muscle of normal zebrafish at 40X-1 magnifications. B) Muscle of resveratrol treated zebrafish exposed to 0.5 µml concentration at 40X-1 magnifications C) Muscle of resveratrol treated zebrafish exposed to 1µml concentration at 40X-1 magnifications D) Muscle of resveratrol treated zebrafish exposed to 1.5 µml concentration at 40X-1 magnifications

Fig. 13.c implies that the histopathological observations in the muscle of resveratrol treated zebrafish made to 1 µml concentration at 40X magnifications. Significant changes were observed in the muscle level of zebrafish in the moderate concentration. Fig. 13.d displays that the histopathological observations in muscle of resveratrol treated zebrafish were subjected to 1.5 µml concentration at 40X magnifications. The most drastic changes can be observed when concentration reaches its highest.

4. DISCUSSION

Zebrafish have become an important model organism for the study of many diseases, including disorders of the metabolism of the bones [27]. The majority of our understanding of how vertebrates respond to short telomeres comes from studies conducted on laboratory mice. It is important to note that mice differ from humans not only in terms of telomere length but also in the processes of cell immortalization and entry into senescence [28,29]. The correlation between indeterminate growth and the timing of senescence is a fascinating phenomenon. Numerous fish species and several other vertebrate species exhibit a unique characteristic of continuous, slow growth throughout their entire lifespan [30,31].

Based on current evidence, it is suggested that the anti-aging effects of resveratrol are linked to its capacity to regulate mitochondria. The biomass and length of the resveratrol-treated zebrafish were investigated on the 30th, 60th and 90th day respectively. The biomass and length have gradually increased from the control group to group III. From the initial day of our analysis, the investigation focused on average biomass and length [32,33].

Based on evidence from morphological and pathophysiological changes observed in various fish species, it is possible to argue that growth and aging can co-occur. This observation challenges the hypothesis that zebrafish exhibit minimal or negligible replicative senescence (the progressive loss of function associated with aging) in vivo [34].

The zebrafish are classified in to four groups and they are treated with resveratrol with various concentrations such as 0.5µ ml, 1.0µ ml and 1.5µ ml for Group I, II and III respectively with the control group. Zebrafish are raised and maintained at specific temperature and calculate the length of the organism. Zebrafish was observed at the initial day, 30th day, 60th day and 90th day.

In the control group, weight was examined and it expresses 0.157g in control group at 0th day. At 30th day, the weight was examined as 0.21g and the weight was 0.326g at 60th day and 0.415g at 90th day. While treated with resveratrol with lowest concentration (0.5µ ml), 0.138g was examined at 0th day. The weight was examined

as 0.211g, 0.332g and 0.51g at 30th, 60th and 90th day respectively. The weight of the zebrafish was determined at 1.0 µ ml concentration as 0.163g and 0.218g at 0th day and 30th day respectively. The biomass was investigated as 0.37g and 0.651g at 60th day and 90th day sequentially. At 1.5 µ ml concentration in the group III, the biomass of the zebrafish was studied as 0.205g at 0th day of our analysis. The biomass was investigated at 30th and 60th day as 0.292g and 0.376g sequentially. At 90th day of our observation, the biomass was 0.668g in this concentration. Based on these investigations, the weight of the zebrafish was gradually increased at all concentrations which were represented in Table 1.

In the control group, the length was examined and it has 2.5cm at 0th day. At 30th day, the length was determined as 2.3cm. At the day of 60 and 90, the length was explored to 2.5cm and 2.9cm. While treated with resveratrol with lowest concentration (0.5µ ml), 2.9 cm length was examined at 0th day. The length was investigated as 2.7 cm, 3.6 cm and 3.9 cm at 30th, 60th and 90th day respectively. The length of the zebrafish was determined at 1.0 µ ml concentration as 3 cm and 2.9 cm at 0th day and 30th day respectively. The length was inspected as 3 cm and 3.9 cm at 60th day and 90th day sequentially. At 1.5 µ ml concentration, the length was determined as 3.2 cm at 0th day. The length was studied as 3cm at 30th day and at 60th day the length was observed as 3.4cm. The length of the zebrafish was 4cm at 90th day. From these observations, the length was gradually increased at each level which were represented in Fig. 3.

The biomass values were determined at 0.42g in control group and 0.55g in group I. The average biomass value has 0.66g and 0.68g at group II and III respectively. The average length was explored 2.90cm in control group and 3.10cm in group I. The average length was determined as 3.90cm and 4.10 cm at 60th and 90th day respectively (Fig. 2. and Fig. 4.).

The highest value for catalase enzyme determined in group II and III as 29.7 and 28.6 respectively. Superoxide dismutase has 5.8 in group II and III and lipid peroxidase has 1.3 in group I. Table 2 represents that the enzymatic activity for various groups. At the 30th day, the results were observed based on the enzymatic activity and it indicates 32.7 and 32.6 Unit/mg. of protein in group 2 and 3 respectively. The superoxide dismutase has 7.5 unit/mg of protein in group 3 and lipid peroxidase has 1.4 in group 2 and 3. These observations clearly showed that the increased concentrations in zebrafish indicate the highest value. The enzymatic assay was performed and the results were assessed at the 60th day of our observation and it indicates that the enhancing activity of enzymes in group II and III. At the 90th day of observation, the result was investigated with highest values in superoxide dismutase at group III. The mode values are observed for enzymatic assay in the highest concentration. Telomerase activity has been observed in adult Xenopus frogs' muscle, heart, and brain [35]. Amphibians, including Xenopus, exhibit ongoing growth even after reaching maturity, and this growth may be facilitated by proportional cell proliferation within their organs [36].

When it comes to the examination of resveratrol's activity, our results closely match their observations. Because of the administration, there has been an increase in the levels and activity of catalase (CAT) and superoxide dismutases (SOD). In a dose-dependent manner, resveratrol administration decreased lipid peroxidation, oxidative and DNA damage, and levels of this gene expression. The levels of reactive oxygen species (ROS), glutathione-S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA), and DNA damage were measured in order to evaluate the toxicity on zebrafish. SOD and GST activity was obviously elevated during the early stages of treatment, but it was ultimately suppressed [37].

Zebrafish have adapted to a moderate concentration of 1.0µ ml, as determined by these investigations. These findings indicate that the resveratrol dosage increases to 1µ ml and 1.5µ ml. The development of the zebrafish's biomass and length was observed on the 90th day of observation. The enzymes lipid peroxidase, superoxide dismutase, and catalase have all revealed the highest protein composition at the same time. Based on the dose-dependent mechanism of resveratrol, it can be concluded that zebrafish have a very gradual process of senescence, as suggested by our studies.

The repetitive DNA sequences are composed to form telomeres which are present at the end of the chromosome [38]. The impact of telomere shortening in a biological system like humans, may evolved to use telomere length as an internal cell division [2]. Klapper et al. suggested

that somatic telomerase activity would be present in animal species exhibiting indeterminate growth. Telomerase activity is closely associated with the ability of cells to proliferate, and there is a significant link between telomere shortening and replicative senescence in human cells that lack telomerase activity. In studies conducted on mice, the introduction of engineered telomerase expression in the adult heart, which naturally lacks intrinsic telomerase activity, induced enhanced proliferative and regenerative capabilities [39]. The mortality rates and various aging parameters can be considerably influenced by local husbandry practices and environmental conditions [4].

The fold variations can obviously be observed in group II when compared with the control group. The telomere length was estimated by the quantifications of telomeres using the delta ct method. Telomere length per diploid cell and telomere length at each chromosome end was observed as 751.79 and 9.95 respectively in group 3. These observations motivate us to investigate further analysis of the functioning of telomeres.

The fold variations are computed after determining the average, ΔCT, ΔΔCT, and 2^ ΔΔCT values at different concentrations. Group 3 exhibits maximum average values for both reference and target genes, while ΔCT, ΔΔCT, and fold variations consistently remain higher. As a result, the highest fold variation was measured at the intermediate concentration, the lowest variance was found at the lowest concentration, and the maximum concentration has a small fold variation.

These findings show that the telomeres of the individuals in the moderate concentration are longer than those in the other concentrations. These findings lead to the conclusion that the zftrt gene is expressed at a higher level in a moderate concentration and that zebrafish have longer telomeres, which protect DNA from damage and increase their lifespan. According to these studies, it is determined that replicative senescence is closely associated with telomere shortening.

According to our histological study, it is found that the shortening of telomeres and dysfunction of mitochondria leads to the breaking of DNA strands and aging occurs in the short duration [40].

Telomere shortening and dysfunction are important causes of aging. Telomeres shield the ends of chromosomes from degradation and the detection of DNA damage response mechanisms. The telomeres are kept from shortening by a special reverse transcriptase called telomerase. The catalysis of telomerase requires the expression of telomerase reverse transcriptase, which is restricted to stem or progenitor cells. Vertebrates undergo telomere destruction during their lifetime, but telomerase expression is insufficient to fully replace it. Aged organisms consequently exhibit telomere dysfunction [41].

The length of time and amount of microparticles in the tissues determined how soon the implant triggered an immune response [42]. According to our study, zebrafish has good immune response after treatment and it prevents the muscle damage.

After administering the drug to zebrafish, it was observed that, although they eventually multinucleated, the red fibers were less divided and more like to those of the controls. Rather, the endomysium's thickness decreased, and it seemed as though the white fibers were enclosed in bigger, more compact myotomes. Rather, even though they still had a large endomysium, they looked smaller than the organisms subjected to the therapy and more like the ones seen in control tissue [43].

Hematoxylin-Eosin staining results in the cell nuclei being stained blue by Hematoxylin, while the cytoplasm and connective tissue are being stained pink by Eosin. Group 3 experienced color shifts, such as blue and pink hues, while the other groups did not. These findings indicate that zebrafish treated with an intermediate concentration of resveratrol retain dye and exhibit positive effects at the muscle level. It is during the peak of concentration that the most significant changes occur.

Understanding the cellular and molecular mechanisms underlying muscle development, function, and diseases may be possible by examining the histopathology of zebrafish muscles. This knowledge may have implications for comprehending disorders related to human muscles and possible treatment approaches. Zebrafish histopathology analysis offers transparency during early developmental stages, fast reproduction, and the ability to study specific

muscle-related mutations through genetic manipulation techniques.

It is proposed that the genes responsible for aging which are present in humans are closely associated with zebrafish. Gene homology and conserved traits with respect to humans can be extended to disease manifestations in order to draw comparisons [44]. According to our study, the activity of natural polyphenol compound resveratrol has been determined for antiaging activity. Natural additives in a range of doses have been used to study animal development, liver histology, and antioxidant effects in zebrafish. The natural additions included plant extract, cashew nut shell liquid, and clove leaf essential oil [45,46].

These findings indicate that zebrafish treated with an intermediate concentration of resveratrol show variations at the muscle level and protect against muscle form damage. The results of this study indicate that zebrafish treated with resveratrol exhibit positive effects on muscular tissue and dye retention at intermediate concentrations. The onset of aging could be delayed if the muscle shields itself from damage. On the basis of these findings, resveratrol is considered a potent antiaging drug that can either stop or postpone the aging process while increasing an organism's longevity.

5. CONCLUSION

This investigation focused on antiaging and zebrafish have been used to study molecular and functional aging. The enzymatic activity indicates the expression of genes in resveratrol treated groups when compared with control. However, the over expression was not observed in dose dependent manner, this might be due to other factors like single time treatment study design. While treating resveratrol, the minor changes are observed during the aging process of zebrafish and our studies suggest that zebrafish shows very gradual senescence. The fold variations are keenly observed in group 2 when compare with control group. The fluctuation in the fold variations was observed in control and other groups. In the lowest concentration (0.5µ ml), it indicates that the lowest variation while in the moderate concentration (1.0µ ml), the highest fold variation was observed. Even in the highest concentration (1.5µ ml), the variation is very less. The amplification plot of hTERT gene expression indicates the magnitude of the normalized fluorescence signal for analysis. From these results, we observed that the telomere length of the treated samples was well protected compared to the control samples. As observed from the ZFTRT gene expression length of the telomere was also slightly protected more than the other treatments. From these investigations, we concluded that telomerase activity is strongly correlated with telomere shortening and replicative senescence. These investigations provide deep insights in antiaging research and it could be possible to help them in future.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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