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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

Computational analysis of superoxide dismutase genes (*sod1, sod2*, and *sod3*) and comprehensive tissue-specific gene expression profiling in Tetraodon (*Tetraodon nigroviridis*)

Tetraodon (*Tetraodon nigroviridis*) süperoksit dismutaz genlerinin (*sod1, sod2* ve *sod3*) in siliko analizi ve dokuya özgü gen ekspresyon profili

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Abstract: The objective of this investigation was to conduct in silico analyses on superoxide dismutase (*sod1, sod2,* and *sod3*) genes in tetraodon (*Tetraodon nigroviridis)*, employing bioinformatics tools, and to assess the gene expressions in various tissues such as the intestine, brain, kidney, liver, muscle, heart, eye, spleen, gills, stomach, ovary, and testis of tetraodon. To achieve this, tissue samples were obtained from both male and female tetraodon, spanning the aforementioned organs, with the purpose of acquiring cDNA. Total RNA was isolated from each tissue, and subsequently, the transcripts of *sod*s genes were assessed using qPCR, while transcript quantities were determined through RT-qPCR. The in silico analyses encompassed the examination of gene structure, conserved gene synteny, phylogenetic tree analyses, and the identification of similarity-identity ratios with other vertebrates. When examining the transcriptional differences between male and female tissues for the Tetraodon *sod1* gene, it was noted that, except for the heart tissue, all other tissues studied (including the liver, intestine, muscle, brain, eyes, spleen, gills, kidney, stomach, and gonads) exhibited significantly higher expression levels in male fish. Examining the results for the *sod2* gene in male and female tetraodon, significant upregulation was observed in the liver, muscle, gills, intestine, ovary, and testis, with no statistical significance in tissues like the intestine, heart, and gonads. Regarding the *sod3* gene in male and female tetraodon, heart, spleen, and stomach tissues did not show statistical significance, but the liver, intestine, gills, kidney, stomach, and gonads exhibited significantly higher expression in male fish ($p<0.05$).

Keywords: Tetraodon, in silico analyses, *sod*s genes, gene expression

Öz: Bu araştırmanın amacı, tetraodon (*Tetraodon nigroviridis*) süperoksit dismutaz (*sod1, sod2* ve *sod3*) genleri üzerinde in siliko analizler yapmak, biyoenformatik araçlar kullanarak bu genlerin çeşitli dokulardaki (bağırsak, beyin, böbrek, karaciğer, kas, kalp, göz, dalak, solungaçlar, mide, yumurtalık ve testis) gen ekspresyonlarını değerlendirmektir. Bu hedefe ulaşmak için, yukarıda belirtilen organlardan cDNA elde etmek amacıyla erkek ve dişi balıklardan doku örnekleri alındı. Her dokudan toplam RNA izole edildi ve ardından *sod*s genlerinin transkriptleri qPCR kullanılarak değerlendirildi. Transkript miktarlarının belirlenmesi amacıya ise RT-qPCR yapıdı. İn siliko analizler, gen yapısının incelenmesini, korunmuş gen sentenisi analizlerini, filogenetik ağaç analizlerini ve diğer omurgalılarla benzerlik-özdeşlik oranlarının tespitini kapsamaktadır. Tetraodon *sod1* geninin erkek ve dişi dokularındaki transkripsiyon farklılıkları incelendiğinde, kalp dokusu dışında çalışılan tüm diğer dokularda (karaciğer, bağırsak, kas, beyin, gözler, dalak, solungaçlar, böbrek, mide ve gonadlar dahil) erkek balıklarda anlamlı derecede daha yüksek ekspresyon seviyeleri gözlendi. Erkek ve dişi tetraodonlarda *sod2* geninin sonuçları incelendiğinde, karaciğer, kas, solungaçlar, bağırsak, yumurtalık ve testiste anlamlı bir yukarı düzenleme gözlendi; bağırsak, kalp ve gonadlar gibi dokularda ise istatistiksel olarak anlamlı bir fark görülmedi. Erkek ve dişi tetraodonlarda *sod3* geni ile ilgili olarak, kalp, dalak ve mide dokuları istatistiksel olarak anlamlılık göstermedi, ancak karaciğer, bağırsak, solungaçlar, böbrek, mide ve gonadlar erkek balıklarda anlamlı derecede daha yüksek ekspresyon sergiledi ($p<0.05$).

Anahtar kelimeler: Tetraodon, in silico analizler, *sod*s genleri, gen ekspresyonu

INTRODUCTION

Superoxide dismutase genes (*sod*s) are essential for antioxidant defense mechanisms, as they catalyze the dismutation of superoxide radicals into less hazardous molecular oxygen and hydrogen peroxide (Chatzidimitriou et al., 2020). These genes are particularly important for teleost fishes, especially Tetraodon , due to their ability to withstand high levels of oxidative stress (Kim et al., 2021). SODs can be classified into three distinct groups based on their redox-active metals: copper/zinc SOD, manganese SOD, and iron SOD (Chen et al., 2022). Superoxide dismutases (SODs; EC 1.15.1.1), considered the first line of defense, are a family of redox-active metalloenzymes that catalyze the conversion of superoxide radicals into molecular oxygen and hydrogen peroxide. It has been demonstrated through homology and phylogenetic data that different SOD isoforms have diverse evolutionary histories within the animal kingdom (Sheng et al., 2014). Superoxide radicals, which are normal byproducts of metabolic oxidation, can cause extensive cellular damage if not neutralized. Both extracellular (secreted) superoxide dismutase (*sod3*) and intracellular superoxide dismutase (*sod1* in the nucleus and cytoplasm, and *sod2* in the mitochondria) play important roles in neutralizing superoxide

radicals (Fujii et al., 2022). Scientific names play a crucial role in biological informatics, providing precision in labeling biodiversity information. However, their utility is limited by semantic ambiguity and syntactic changes that don't always reflect taxonomic modifications (Remsen, 2016). This is evident in the genus Tetraodon, a group of pufferfish species used in bioinformatics research. The Tetraodon nigroviridis genome, characterized by its compact size and reduced intergenic and intronic sequences, has been analyzed for its repeat content and organization (Roest Crollius et al., 2000). Pufferfish genomes, including Tetraodon, are valuable for comparative genomics due to their small size yet complex structure, with preserved gene structures despite reduced intron sizes (Koop and Nadeau, 1996). Tetraodon (*Tetraodon nigroviridis*) is a remarkable species due to its unique biological characteristics, particularly its ability to withstand high levels of oxidative stress (Wang et al., 2016). Investigating the genetic basis of antioxidant defense in Tetraodon can offer valuable insights into their adaptation mechanisms and contribute to a better understanding of the evolution of *sod* genes in aquatic vertebrates (Stump et al., 2018; Ahn et al., 2018). *sod* genes play a crucial role in teleost fish adaptation mechanisms by helping maintain redox balance within cells, ensuring that oxidative stress does not lead to cellular damage in response to environmental perturbations or pathogenic infections (Kim et al., 2021). Research on the river pufferfish, *Takifugu obscurus*, has identified a robust antioxidant system in its liver, with genes such as catalase, glutathione reductase, and superoxide dismutase being significantly induced in response to cadmium exposure (Kim et al., 2010). Similarly, the Japanese pufferfish, *Takifugu rubripes*, has been found to possess functional NADPH oxidase components, which play a crucial role in host defenses against microbial infection (Inoue et al., 2004). The compact genome of the Japanese pufferfish has also facilitated the isolation and characterization of serine/threonine phosphatase genes (Koh et al., 1997). Furthermore, the identification of novel genes related to tetrodotoxin intoxication in pufferfish, such as fibrinogen-like proteins, suggests a potential role in detoxification processes (Lee et al., 2007). The green pufferfish, *Tetraodon nigroviridis*, is an important genetic model organism, and various studies have been conducted on its molecular characteristics, gene expression, and development (Rothenburg et al., 2008; Watson et al., 2009; Bayır and Arslan, 2020; Bayır, 2020).

In this study, we aimed to gain valuable insights into the adaptation mechanisms and evolution of sod genes in Tetraodon, by understanding the genetic basis of antioxidant defense. To achieve this goal, we performed an in silico analysis of the *sod1, sod2*, and *sod3* genes in Tetraodon, examining their gene expressions across various tissues. Through the analysis of genomic data, our objective was to reveal the structural and functional aspects of the *sod/SOD* genes and their crucial role in maintaining redox balance in this ecologically significant species.

MATERIALS AND METHODS

Fish sampling and experimental designs

The material for the study consists of 3 female and 3 male Tetraodon (*Tetraodon nigroviridis*) obtained from the Faculty of Fisheries at Atatürk University. The molecular analyses were conducted at the Agricultural Biotechnology Laboratory. Tissue samples, including intestines, brain, kidneys, muscles, liver, heart, eyes, spleen, gills, stomach, and gonads, were collected from all the fish and preserved in 1 ml RNA later in 2 ml Eppendorf tubes at +4°C for 24 hours and then at -80°C until the day of analysis. Before sampling, the Tetraodon were anesthetized with clove oil. The entire study was conducted in accordance with the Local Ethics Committee for Animal Experiments at Atatürk University.

RNA isolation and cDNA synthesis and real-time PCR (qPCR) analysis

Tissue samples were initially extracted from RNAlater and then placed into nuclease-free tubes with 1 ml of trizol reagent (Life Technologies) for homogenization. The Trizol protocol was employed to isolate RNA. Subsequently, RNA concentrations were assessed using a Nanodrop 8000 spectrophotometer, and the quality of total RNA was determined through agarose gel-electrophoresis. To prevent genomic contamination, all RNAs underwent DNase treatment (DNase I, Amplification Grade, Life Technologies) prior to cDNA synthesis. The High-Capacity cDNA Reverse Transcription Kit (Life Technologies) was utilized for the cDNA synthesis.

Quantitative PCR was conducted using the Rotor-Gene 6000 thermal cycler system (Qiagen GmbH, Düsseldorf, Germany) and the QuantiTect SYBR Green PCR kit (Qiagen) to determine the tissue-specific distribution (copy number/µL) of Tetraodon target genes (*sod1, sod2,* and *sod3*) and reference genes (*rpl7* and *rpl13a*). Each Quantitative PCR reaction for a tissue sample, including a negative control, comprised 20µl (10 µL SYBR Green, 4 µL forward and reverse primer, 5 µL nuclease-free water, and 1 µL cDNA). The RT-qPCR steps involved initial denaturation (95.0 °C for 15 min), followed by 40 cycles of denaturation (95.0°C for 20 s), primer annealing at the optimum temperature for each primer (Table 1) for 30 s, and elongation (72.0°C for 30 s). The mRNA transcript levels of *sod1, sod2,* and *sod3* genes in Tetraodon tissues were normalized to *rpl7* and *rpl13a* to assess tissue-specific distribution post qPCR. The gene expression levels were reported relative to the mean value of the control groups (Anderson and Elizur, 2012).

Primer optimisation

The forward and reverse primers were created using NCBI Primer-BLAST for the real-time quantitative PCR (qPCR) amplification of Tetraodon target genes (*sod1, sod2,* and *sod3*) as well as reference genes (*rpl7* and *rpl13a*) (Table 1). The primers were designed based on an exon-exon junction model to prevent the PCR amplification of products originating from any contaminating heterogeneous nuclear

RNA (hnRNA) or genomic DNA. The lyophilized primers were ordered and then reconstituted in TE buffer (10mM Tris, 1mM EDTA, pH 8.0) in a manner that achieved a stock concentration of 100 pmol/µl for each primer.

Table 1. Primer sequences for Tetraodon genomic (*sod1, sod2,* and *sod3*), Target Genes (*sod1, sod2,* and *sod3*), and reference genes (*rpl7* and *rpl13a*)

Tetraodon		Forwardprimer(5´→3´)	Reverseprimer(5´→3´)	Tm(°C)
sod1	Target	ATGTTTGGTTTTCCAGCAAGCGCAG	CGGGGACACGGTAGTTGTAG	60.6
sod2	Target	ACAGCGTTCGCCTCTGCTGTC	CTCTTTTTGGCAGTTTGGAGACG	61.4
sod3	Target	CGTTGACGATGCGTCTGCAC	GCCGGATACAAAGATGGAAT	61.5
rpl7	Reference	CGAGAAAAAGGCCCGCAAG	GGCTGACACCGTTGATACCT	59.7
rpl13a	Reference	TCCACCCTACGACAAGAGGAA	GTACTTCCAGCCAACCTCAT	60.20
sod1	Genomic	ATGTTTGGTTTTCCAGCAAGCGCAG	CTGTTTACTGAGTGATGCCGATG	61
sod2	Genomic	ACAGCGTTCGCCTCTGCTGTC	CTACTTTTTGGCAGTTTGGAGACG	63.20
sod3	Genomic	CGTTGACGATGCGTCTGCAC	GCCGGATACAAAGATGGAAT	60.5

The process of identifying and determining the structure of Tetraodon *sod1, sod2,* and *sod3* genes

The Ensembl database was used for bioinformatic identification of *sod1, sod2* and *sod3* genes. To confirm the accuracy of the obtained cDNA from this database, a BLAST search was performed in the NCBI database (https://www.ncbi.nlm.nih.gov/). This study revealed that the superoxide dismutase gene, utilized as the target gene, possesses three isoforms, specifically *sod1, sod2,* and *sod3*.The Ensembl gene IDs and amino acid numbers are provided in the Table 2.

The similarity and identity rates of Tetraodon *sod1, sod2*, and *sod3* genes with those of other teleost fish and vertebrates were determined using the BLOSUM62 matrix algorithm (Gromiha 2010). Protein sequences synthesized by *sods*/*SOD*s genes from Tetraodon (*Tetraodon nigroviridis*), fugu (*Fugu rubripes*), stickleback (*Gasterosteus aculeatus*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), goldfish (*Carassius auratus*), human (*Homo sapiens*), and mouse (*Mus musculus*) were utilized for calculating similarity-identity rates. This analysis was performed using the BioEdit program, and the results are presented in Figures 1, 2 and 3.

Table 2. Tetraodon *sod1, sod2,* and *sod3* genes with ENSEMBL accession numbers and amino acid numbers

Gene	Organism	Ensembl gene ID	Amino acit numbers
sod1	Tetraodon	NSTNIT00000013030.1	179
sod2	Tetraodon	ENSTNIT00000015459.1	225
sod3	Tetraodon	ENSTNIT00000015540.1	209

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Tetraodon Sod1     1   ------------------------------------------MFGFPASAVLPCVSFLEV
Fugu Sod1          1   ------------------------------------------------------------
Stickleback Sod1   1   ------------------------------------------------------------
Zebrafish Sod1     1   ------------------------------------------------------------
Medaka Sod1        1   ------------------------------------------------------------
Mouse Sod1         1   ------------------------------------------------------------
Human SOD1         1   ------------------------------------------------------------
Goldfish Sod1      1   MKAFRQVQNLGIILKSLTSLIVSRQTLTQPTSTSLFCLLIGQFDSRSS.FIKLFF.LFWL

Tetraodon Sod1    19   TT----AKMVIKAVCVLKGAGETSGTVYFEQQDEKAPVKLTGEIKGLTAGEHGFHVHAFG
Fugu Sod1          1   --------.AM.........D.........--.S............P..........
Stickleback Sod1   1   --------..L........S..V....H....NGD....V..K.S...P.D.......
Zebrafish Sod1     1   --------..N........T..VT.....N.EG..K...V....T...P.K.......
Medaka Sod1        1   --------..L........T...N.V.N...ESDS....V........P.K....I.VY.
Mouse Sod1         1   --------.AM........D.PVQ..IH...KASGE..V.S.Q.T...E.Q......QY.
Human SOD1         1   --------.AT........D.PVQ.IIN...KESNG...VW.S.....E.L......E..
Goldfish Sod1     61   GHNSQFDS.AM........T..VI.N......N.TSS...S.K.T...P.K.........

Tetraodon Sod1     75  DNTNGCISAGPHYNPHNKTHAGPNDENSLKRHVGDLGNVTAEADQIAKIDITDSVISLHG
Fugu Sod1          51  .......................T.AD---..L........G..N......K..MLT.T.
Stickleback Sod1   53  ......V.............G..R...---...........GE.KV.N.N.E.KH.T.T.
Zebrafish Sod1     53  ............F...D...G..T.SV---...........D.SGV...E.E.AMLT.S.
Medaka Sod1        53  ......V.....F..Y..N.G..E.AE---...........GDNNV.......KL.R.S.
Mouse SOD1         53  ...Q..T.....F...S.K.G..A..E---...........GK.GV.NVS.E.R....S.
Human SOD1         53  ...A..T.....F..LSRK.G..K..E---...........DK.GV.DVS.E......S.
Goldfish Sod1     121  ............F..FG.D.G..T.SD---.........I.DDNGV.....E.KIVT.L.

                                                                 Similarity(%)  Identity(%)
Tetraodon Sod1    135  KFSIIGRTMVIHEKADDLGKGGNEESLKTGNAGGRLACGVIGITQ     100          100
Fugu Sod1         108  PY.........................................       74           79
Stickleback  Sod1 110  Q.....................DD...................A.     69           77
Zebrafish Sod1    110  QH...........E.............................       68           75
Medaka Sod1       110  PD..V...V.V...V.........D.........A.........A.     63           73
Mouse SOD1        110  EH........V...Q...........T......S.........A.     60           68
Human SOD1        110  DHC.....L.V..............T......S.........A.     59           66
Goldfish Sod1     178  QH...........E..................S...........      52           60
```

Figure 1. The similarity-identity ratios between the protein sequence of the Tetraodon (*Tetraodon nigroviridis*) *sod1* gene and the Sod1 protein sequences of some other vertebrates. (The dots in the table indicate similarities, while short dashes represent missing amino acids)

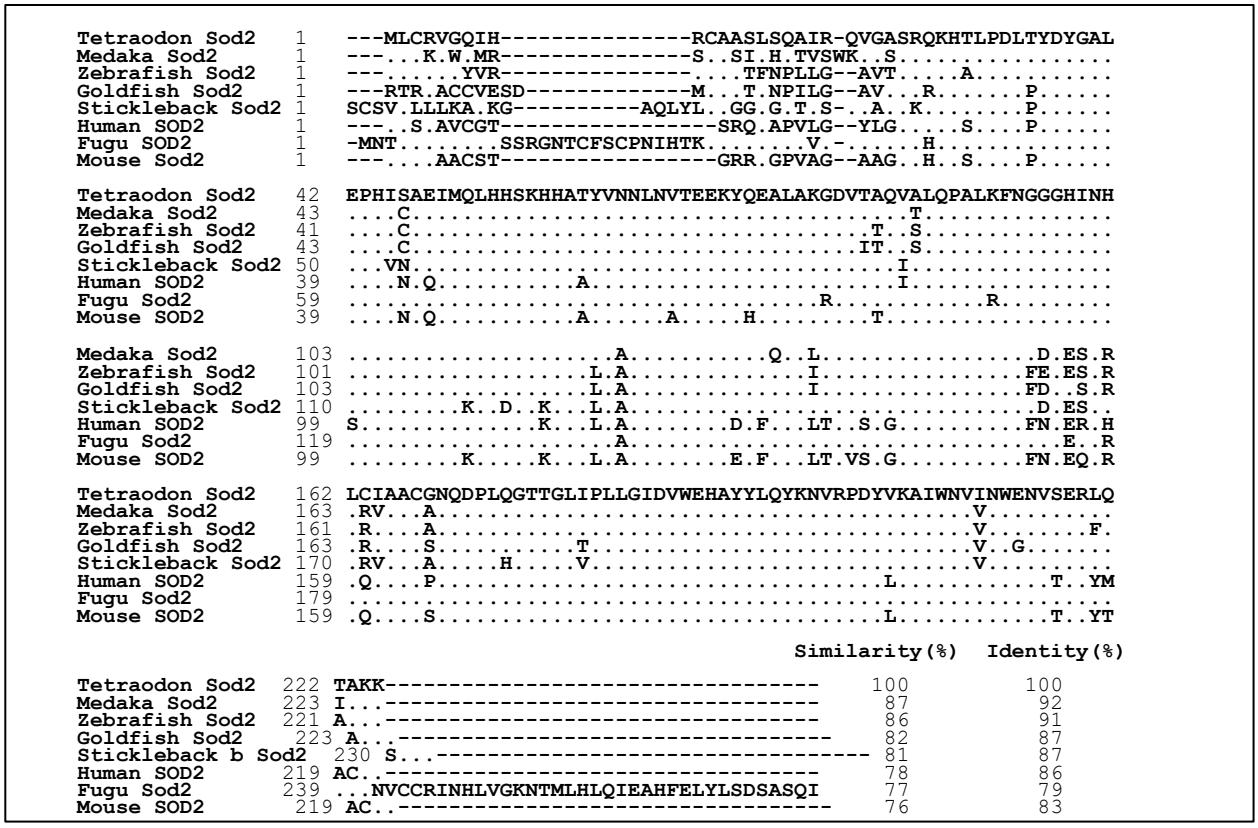

Figure 2. The similarity-identity ratios between the protein sequence of the Tetraodon (*Tetraodon nigroviridis*) *sod2* gene and the Sod2 protein sequences of some other vertebrates. (The dots in the table indicate similarities, while short dashes represent missing amino acids)

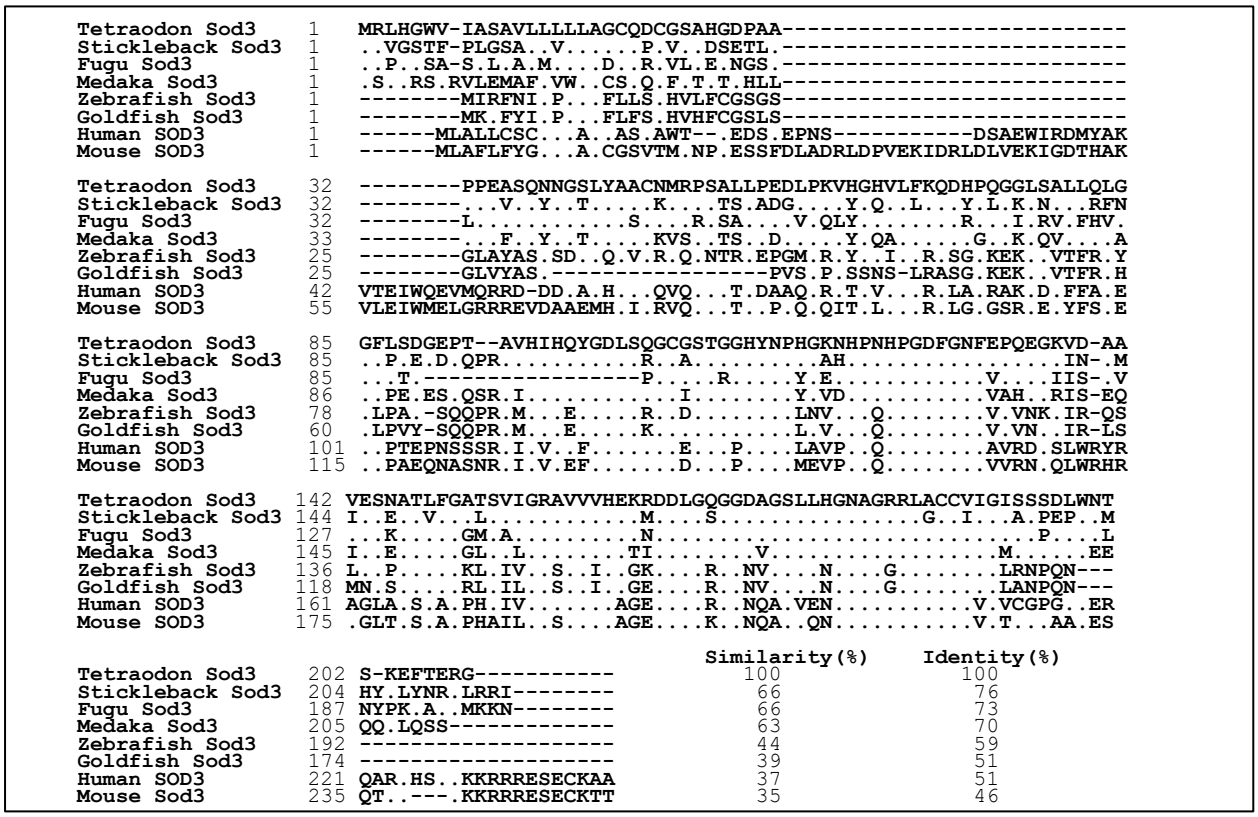

Figure 3. The similarity-identity ratios between the protein sequence of the Tetraodon (*Tetraodon nigroviridis*) *sod3* gene and the Sod3 protein sequences of some other vertebrates. (The dots in the table indicate similarities, while short dashes represent missing amino acids)

The determination of the nucleotide sequences of the *sod1, sod2*, and *sod3* genes in the Tetraodon (*Tetraodon nigroviridis*) has been accomplished. The nucleotide sequences, including intron and exon sequences, of the *sod1, sod2,* and *sod3* genes in the pufferfish have been identified in the ENSEMBL database.

The acquisition of cDNA was facilitated using the ENSEMBL database. The nucleotide sequences have been designed to depict the exons, introns, amino acids synthesized by the exons, 5' and 3' ends, TATA box, poly-A signal, and stop codon of the *sod1, sod2*, and *sod3* genes (Figures 4, 5 ,6).

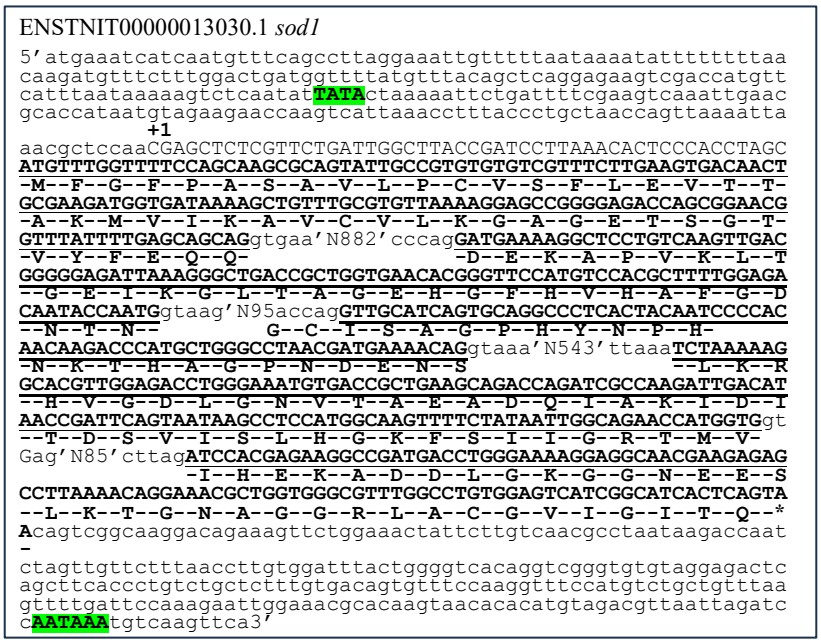

Figure 4.. Exon-intron organization of the Tetraodon (*Tetraodon nigroviridis*) *sod1* gene*

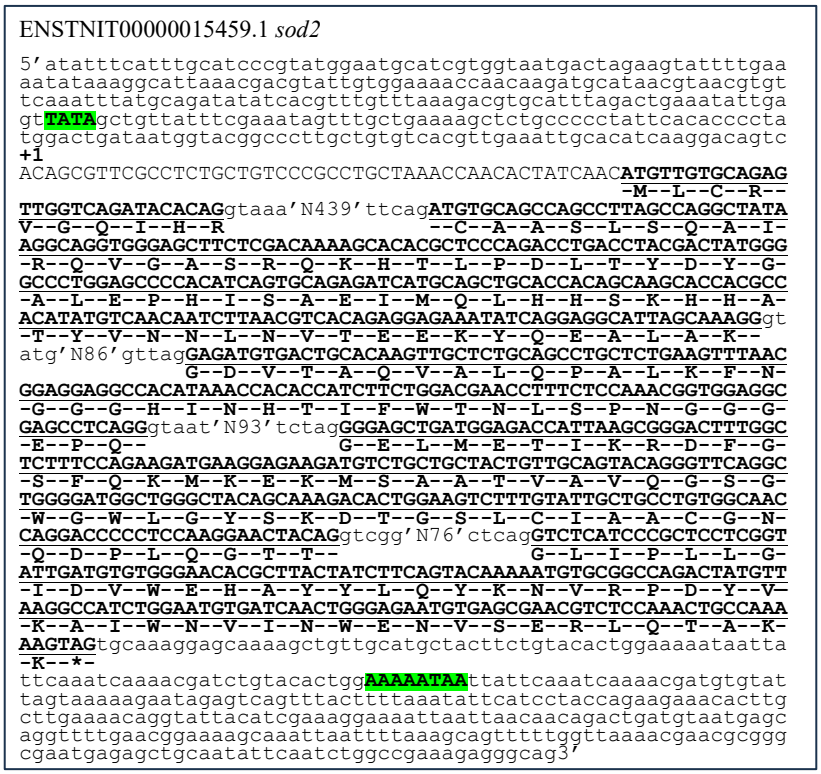

Figure 5. Exon-intron organization of the Tetraodon (*Tetraodon nigroviridis*) *sod2* gene*

ENSTNIT00000015540.1 *sod3*

```
5'ttaaacaagaacaggctattgccttttatttatttatcaaatattgcctaatttctat
ggatagaaaTATAttttttaaagacatgggagctttaactggtcgtgttacctttcatgga
aagcaggtttctttttgctgttgacggtttctttttttaaaaagcagtcattaaaaatta
aacattgcaatttctcataaaaaacatgtgtgtaaacaaagtgtccacccttggaaaatg
agccccaccttcatgtttcattggtctgctcagaacagcgagcagcggtcggattgatcc
+1
AGACTGAAAGTCCACCTTCGTTGACGATGCGTCTGCACGGgtaag'N785'ttcagGTGG
                          -M--R--L--H--G                --W-
GTGATCGCGTCGGCAGTGCTGCTGCTTCTGCTGGCCGGTTGTCAAGATTGCGGCTCAGCT
V--I--A--S--A--V--L--L--L--L--L--A--G--C--Q--D--C--G--S--A--
CACGGTGACCCTGCAGCTCCGCCGGAGGCCTCTCAGAACAATGGCAGCCTGTATGCGGCC
-H--G--D--P--A--A--P--P--E--A--S--Q--N--N--G--S--L--Y--A--A-
TGCAACATGAGACCCAGCGCCTTGCTGCCAGAGGACCTGCCCAAAGTGCACGGTCACGTG
-C--N--M--R--P--S--A--L--L--P--E--D--L--P--K--V--H--G--H--V-
CTGTTCAAGCAGGACCACCCTCAGGGAGGACTCTCGGCCCTCCTTCAGCTTGGCGGCTTT
-L--F--K--Q--D--H--P--Q--G--G--L--S--A--L--L--Q--L--G--G--F-
CTCAGCGACGGCGAGCCCACGGCCGTCCACATCCATCAGTACGGGGACCTGAGCCAGGGG
-L--S--D--G--E--P--T--A--V--H--I--H--Q--Y--G--D--L--S--Q--G-
TGCGGCTCCACCGGTGGGCACTACAACCCACACGGCAAAAACCACCCCAACCACCCCGGA
-C--G--S--T--G--G--H--Y--N--P--H--G--K--N--H--P--N--H--P--G-
GACTTTGGTAACTTTGAGCCTCAGGAGGGGAAGGTCGACGCCGCGGTAGAGTCAAACGCC
-D--F--G--N--F--E--P--Q--E--G--K--V--D--A--A--V--E--S--N--A-
ACGCTCTTTGGAGCGACGTCTGTGATCGGAAGGGCAGTGGTGGTCCACGAGAAGAGAGAT
-T--L--F--G--A--T--S--V--I--G--R--A--V--V--V--H--E--K--R--D-
GACCTGGGCCAGGGTGGAGACGCCGGGAGCCTCCTGCACGGAAACGCAGGACGGAGGCTT
-D--L--G--Q--G--G--D--A--G--S--L--L--H--G--N--A--G--R--R--L-
GCCTGCTGCGTTATTGGAATTTCCTCTTCCGATCTGTGGAACACCTCCAAGGAGTTTACA
-A--C--C--V--I--G--I--S--S--D--L--W--N--T--S--K--E--F--T-
GAAAGGGGGTAAaaaatacagtaatttacatgcaaaacataaacagctgagacggaggtt
-E--R--G--*-
ctttaagaaaacgtcgcttgagtatcttttggttttaaagatgttcagcagaaaacagca
gctgtgccatcgcctacgactcacctcatctaccaacgtctatcagagtttgacaagcta
cggtggtgattctgtcctgctcagcgctgttgatcaatgctttacacatatggtttcttc
agaggtaggaagATAAAAgaaaagaggtttcatccgacagaaactgtcggaaaaaccgcc
gttattgctacaag3'
```

Figure 6. Exon-intron organization of the Tetraodon (*Tetraodon nigroviridis) sod3* gene*

*The exons of the Tetraodon superoxide dismutase (*sod1, sod2,* and *sod3*) genes are indicated in uppercase letters. In transcription, the starting point is denoted as +1, and the 5' and 3' sequences are indicated in lowercase letters. The TATA box and poly-A signal (ATAAAA) are highlighted in green and represented in uppercase letters.

Phylogenetic analysis

The alignment of Tetraodon *sod*s genes was conducted using the CLUSTALW algorithm (Thompson et al., 1994) within the BioEdit software. The phylogenetic tree constructed using the Maximum Likelihood Method (Felsenstein, 1981) includes the following organisms, protein sequences, and accession numbers for the *sod1* gene: Tetraodon ENSTNIT00000013030.1, zebrafish (*Danio rerio*) ENSDART00000064376.5, Japanese goldfish (*Carassius auratus*) ENSCART00000041002.1, Medaka (*Oryzias latipes*) ENSORLT00000027902.1, human (*Homo sapiens*) ENST00000270142.11, and mouse (*Mus musculus*) ENSMUST00000023707.11 protein sequences were used. For the *sod2* gene: Tetraodon (*Tetraodon nigroviridis*) ENSTNIT00000015459.1, zebrafish (*Danio rerio*) ENSDART00000062556.4, Japanese goldfish (*Carassius auratus*) ENSCART00000055705.1, Medaka (*Oryzias latipes*) ENSORLT00000016614.2, human (*Homo sapiens*) ENST00000337404.8, and mouse (*Mus musculus*) ENSMUST00000007012.6 protein sequences were used. For the *sod3* gene: Tetraodon (*Tetraodon nigroviridis*) ENSTNIT00000015540.1, zebrafish (*Danio rerio*) ENSDART00000112150.4, Japanese goldfish (*Carassius auratus*) ENSCART00000099621.1, medaka (*Oryzias latipes*) ENSORLT00000024189.2, human (*Homo sapiens*) ENST00000382120.4, and mouse (*Mus musculus*) ENSMUST00000101208.6 protein sequences were used. ENSEMBL and NCBI databases were used for data acquisition (Figure 7).

Conserved gene synteny

The conserved gene synteny has been manually designed using the ENSEMBL database, and for this purpose, the chromosomes and chromosomal regions where the superoxide dismutase *(sod1, sod2,* and *sod3*) gene is located in Tetraodon have been recorded. The *sod1* gene is found on the chromosome 10 in zebrafish, on the chromosome 7 in Tetraodon, and on the chromosome 21 in human. It has been observed that the genes *ltn1, paxbp1, grik1, tiam1, sod1, scaf4, synj1, cxadr* are also located on the same chromosomes in zebrafish, Tetraodon, and humans. The *sod2* gene is identified on the chromosome 20 in zebrafish, on the chromosome 14 in Tetraodon, and on the chromosome 6 in humans. Other genes found on the same chromosomes as *sod2* in these three organisms include *kif25, acat2, wtap, sod2, slc22a16, cep57l1, sesn1, snx3.* Finally, for *sod3*, it is located on the chromosome 1 in zebrafish, on

the un_random chromosome in Tetraodon, and on the chromosome 4 in humans. Other genes found on the same chromosomes as *sod3* in these three organisms include *fgb, fga, exosc9, fabp2, vegfc, ing2, sod3, clgn*. A conserved gene synteny has been created based on the common genes present in the genomes of these three organisms and their chromosomal locations. These findings allowed us to create a conserved gene synteny map that showed the relationship among the *sod1/SOD1, sod2/SOD2* and *sod3/SOD3* genes of Tetraodon, zebrafish, and human (Figure 8).

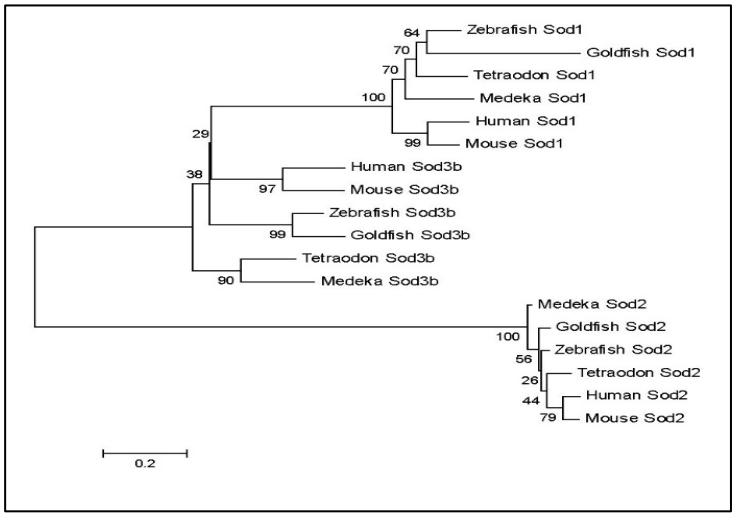

Figure 7. The phylogenetic relationships of the *sod1, sod2*, and *sod3* genes of Tetraodon (*Tetraodon nigroviridis*) with those of other teleost fishes and tetrapods were examined to understand their evolutionary context

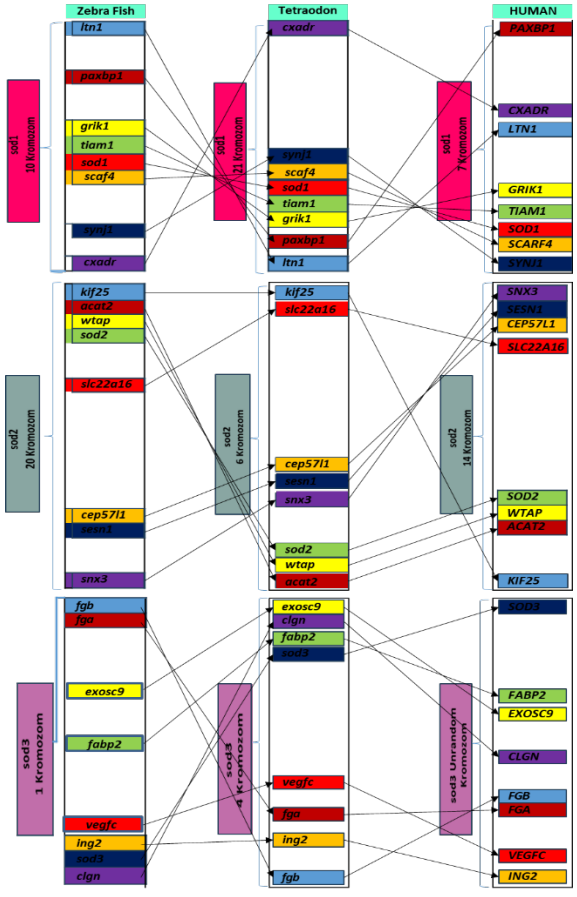

Figure 8. Conserved gene synteny of the Tetraodon *sod1, sod2,* and *sod3* genes with *sod1/SOD1, sod2/SOD2*, and *sod3/SOD3* genes from zebrafish and human

Statistical analysis

In this research study, the results normalized after qPCR application were evaluated through statistical analysis. The statistical analysis utilized the SPSS statistical program, and differences were determined to be statistically significant ($P<0.05$) by applying ANOVA (Duncan's multiple comparison test) to the results (SPSS 1996).

RESULTS

Gender-specific expression of *sod1, sod2* and *sod3* Genes in different tissues of Tetraodon (*Tetraodon nigroviris*)

In this study, tissue-specific distributions of the *sod1, sod2,* and *sod3* genes were determined in female and male Tetraodon through qPCR transcription measurements (Figure 9, 10, and 11). For the female Tetraodon, tissue-specific distribution of the *sod1* gene was determined as follows: liver 28.74 ± 1.21; intestine 13.88 ± 0.5; muscle 5.09 ± 0.42; brain 2.72 ± 0.24; heart 3.41 ± 0.32; eye 1.88 ± 0.1; spleen 1.52 ± 0.1; gill 6.37 ± 0.85; kidney 1.62 ± 0.11; stomach 2.01 ± 0.14; ovary 7.16 ± 0.96. For male Tetraodon, tissue-specific distribution of the *sod1* gene was determined as follows: liver 40.46 ± 3.22; intestine 22.68 ± 1.63; muscle 8.86 ± 1.04; brain 5.14 ± 0.96; heart 3.79 ± 0.55; eye 3.08 ± 0.42; spleen 3.64 ± 0.88; gill 10.91 ± 1.45; kidney 4.08 ± 1.27; stomach 3.84 ± 0.99; testis 10.84 ± 1.08. The results showed that the liver had higher gene expression compared to all other tissues, and the intestine, ovary, and gill had significantly lower the *sod1* gene expression compared to the liver, while all other tissues had significantly higher expression. When examining the transcriptional differences between male and female tissues for the Tetraodon *sod1* gene, it was observed that the intestine, gill, kidney, stomach, muscle, and gonads were significantly higher in male fish, but the differences among other tissues were not statistically significant (Figure 9).

In female tetraodon, tissue-specific distribution of the *sod2* gene is determined as follows: liver 32.93 ± 3.01; intestine 19.29 ± 1.99; muscle 16.64 ± 1.52; brain 4.91 ± 1.01; heart 5.6 ± 1.44; eye 3.79 ± 0.62; spleen 3.77 ± 0.85; gill 25.46 ± 1.89; kidney 2.85 ± 0.55; stomach 2.89 ± 0.22; ovary 13.46 ± 1.07. For male tetraodon, tissue-specific distribution of the *sod2* gene is determined as follows: liver 35.54 ± 3.08; intestine 19.72 ± 2.11; muscle 12.07 ± 1.19; brain 4.14 ± 0.09; heart 5.32 ± 1.11; eye 2.07 ± 0.52; spleen 1.99 ± 0.28; gill 30.63 ± 3.24; kidney 4.81 ± 0.35; stomach 4.17 ± 0.96; testis 14.43 ± 1.33.

When examining the results for both male and female tetraodon for the *sod2* gene, it is observed that the liver, muscle, gill, intestine, ovary, and testis have significantly higher the *sod2* gene expression compared to all tissues, while the differences among the intestine, heart, and gonad tissues are not statistically significant (Figure 10).

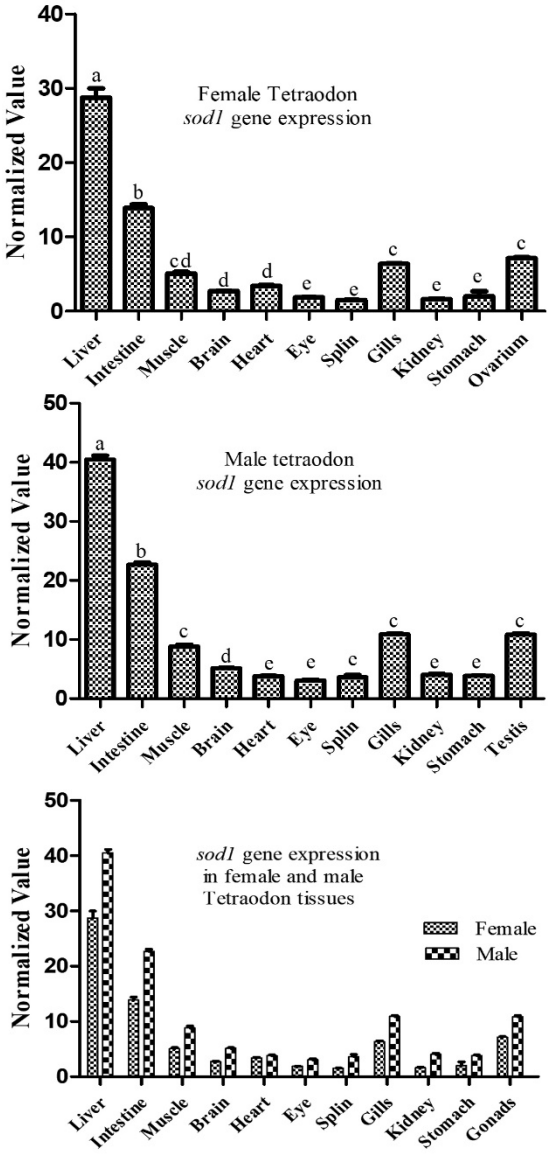

Figure 9. Tissue-specific expression of the *sod1* gene in female and male Tetraodon

For female tetraodon, the tissue-specific distribution of *sod3* gene is determined as follows: liver 33.04 ± 1.17; intestine 23.2 ± 1.11; muscle 6.82 ± 0.09; brain 1.85 ± 0.01; heart 2.99 ± 0.02; eye 1.05 ± 0.01; spleen 1.42 ± 0.01; gill 16.70 ± 2.07; kidney 0.94 ± 0.01; stomach 1.05 ± 0.02; ovary 10.85 ± 0.99. For male tetraodon, the tissue-specific distribution of the *sod3* gene is determined as follows: liver 42.31 ± 2.71; intestine 24.76 ± 2.01; muscle 5.70 ± 1.11; brain 2.67 ± 0.08; heart 3.90 ± 0.08; eye 2.12 ± 0.06; spleen 1.6 ± 0.04; gill 19.17 ± 1.10; kidney 1.60 ± 0.05; stomach 1.29 ± 0.04; testis 20.67 ± 1.27. When examining the results for both male and female tetraodon for the *sod3* gene, it is observed that the heart, spleen, and stomach tissues do not show statistically significant differences, but all other tissues are significantly higher in male tetraodon (Figure 11).

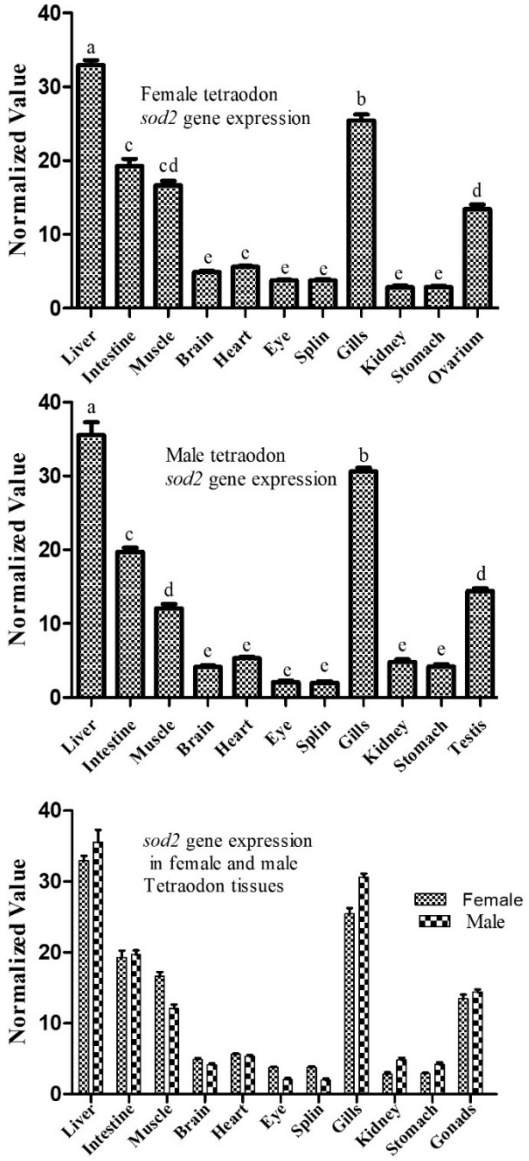

Figure 10. Tissue-specific expression of the *sod2* gene in female and male Tetraodon

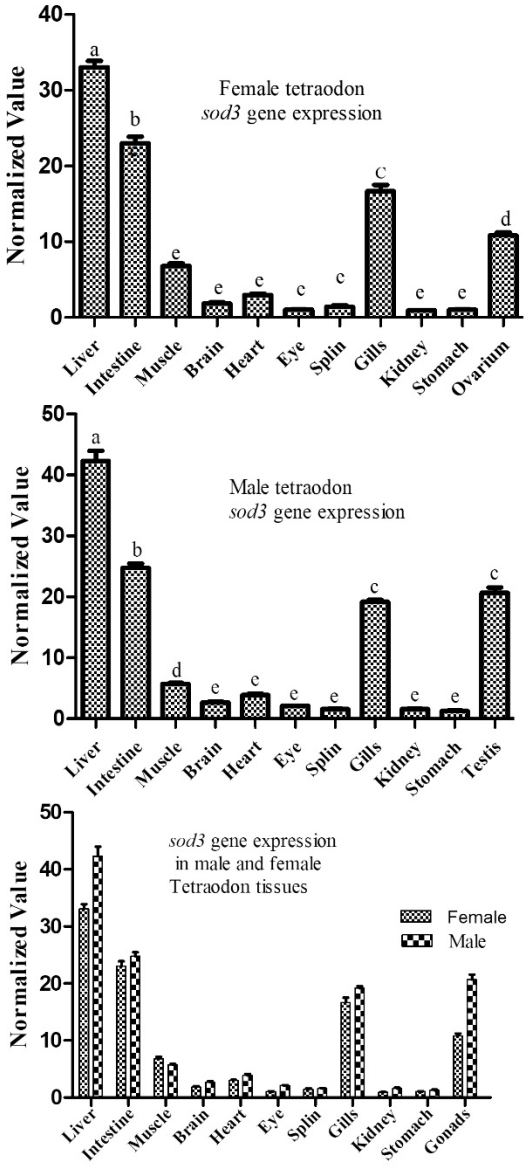

Figure 11. Tissue-specific expression of the *sod3* gene in female and male Tetraodon

3.2. Bioinformatics studies of *sod1, sod2,* and *sod3* genes in Tetraodon (*Tetraodon nigroviridis*)

The bioinformatics studies conducted for the characterization and identification of *sod1, sod2,* and *sod3* genes in tetraodon aim to establish foundational information for the development of contemporary strategies to mitigate the adverse effects of oxidative stress in both fish and other vertebrates. The analysis revealed that not only tetraodon but also other fish species such as zebrafish, goldfish, medaka, and stickleback exhibited a structure for *sod1* and *sod2* genes consisting of 5 exons and 4 introns, while the *sod3* gene comprised 2 exons and 1 intron, demonstrating a highly conserved exon-intron organization. Using CLUSTAL W for sequence alignment analysis (Thompson et al., 1994), it was noted that the levels of polypeptide identity and similarity between tetraodon and various species including zebrafish, medaka, goldfish, stickleback, fugu, mouse, and human were notably elevated. Furthermore, the analysis indicated that the tetraodon *sod1* gene exhibited the highest similarity (74%) and identity (79%) rates with the fugu, *sod2* gene exhibited the highest similarity (87%) and identity (92%) rates with the medaka, while the *sod3* gene displayed the highest similarity (66%) and identity (76%) rates with stickleback.

The phylogenetic relationship can be seen in the tree created using protein sequences of tetraodon (*Tetraodon nigroviridis*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), goldfish (*Carassius auratus*) human (*Homo sapiens*), and mouse (*Mus musculus*) according to the maximum-likelihood method using the MEGA11 program. It was observed that the *sod1*, *sod2*, and *sod3b* genes were clustered in different regions (Figure 7).

DISCUSSION

Tissue-specific transcriptional activity of *sod1, sod2,* and *sod3* genes in male and female Tetraodon (*Tetraodon nigroviridis*)

Fish, due to their adaptation to a broad range of habitats and stressful environmental conditions associated with life strategies, tend to be exposed to harmful reactive oxygen species (ROS)-mediated oxidative stress conditions (Carney Almroth et al., 2015; Wang et al., 2016; Chatzidimitriou et al., 2020). Therefore, understanding how fish have evolved to cope with oxidative stress, including normal cellular metabolism, environmental changes, and/or pathogenic infections through various mechanisms, is particularly intriguing.

The expression of the *sod1, sod2, and sod3* genes in different tissues of *Tetraodon nigroviridis* is influenced by gender, with significant differences observed in the liver, intestine, gill, and kidney (Isensee and Noppinger, 2007). This gender-specific expression is consistent with the sexually dimorphic gene expression observed in mammalian somatic tissue (Guan et al., 2000). The role of these genes in reproductive function is further supported by the subfertility of female mice lacking SOD1 (Matzuk et al., 1998). The response of these genes to environmental stressors, such as the down-regulation of *sod* genes in platyfish exposed to diazinon, highlights their potential as biomarkers for environmental toxicity (Bayır and Özdemir, 2023). Uzun and Bayır (2023) investigated the expression differences of the *gsr* and *g6pd* genes, which are antioxidant enzyme genes, between genders in zebrafish. They found that the expression of the *gsr* gene was significantly higher in the liver, intestine, heart, eye, gills, and reproductive organs of male zebrafish compared to female fish. Additionally, they observed that the transcription of the *g6pd* gene was significantly higher in the male liver, intestine, muscle, brain, eye, gills, kidney, stomach, and reproductive organs. When examining the transcriptional differences between male and female tissues for the Tetraodon *sod1* gene, it was noted that, except for the heart tissue, all other tissues studied (including the liver, intestine, muscle, brain, eyes, spleen, gills, kidney, stomach, and gonads) exhibited significantly higher expression levels in male fish. Upon analyzing the *sod2* gene results in male and female Tetraodon, a significant increase in expression was observed in the liver, intestine, muscle, gills, spleen, eyes, kidneys, and stomach, with no notable statistical significance in tissues such as the intestine, heart, and gonads. Regarding the *sod3* gene in male and female Tetraodon, tissues like the heart, spleen, and stomach showed no statistical significance, yet the liver, intestine, gills, kidneys, stomach, and gonads displayed markedly higher expression levels in male fish.

Superoxide dismutases (SODs) play crucial roles in antioxidant defense across various organisms. In fish, SOD genes exhibit differential expression patterns between sexes and tissues (Ferrão et al., 2024; Bayır and Özdemir, 2023). The analysis of transcriptional differences between male and female tissues for the Tetraodon *sod1* gene revealed intriguing findings. Notably, except for the heart tissue, all other tissues studied displayed significantly higher expression levels in male fish. This observation suggests a potential sex-dependent regulation of the *sod1* gene expression across various tissues in Tetraodon. This phenomenon is observed in various organisms, including fish, where superoxide dismutase (SOD) genes exhibit differential expression between sexes (Bayır and Özdemir, 2023). In cichlid fishes, sex-specific gene expression is more pronounced in gonads than in the brain, with a trend towards male-biased expression, particularly in mouth-breeding species (Böhne et al., 2014). The higher expression levels of the *sod1, sod2,* and *sod3* genes in male fish across multiple tissues could be indicative of several underlying factors. Firstly, it may reflect inherent physiological differences between male and female Tetraodon individuals, possibly related to their reproductive roles or metabolic demands. For instance, male fish may require elevated antioxidant defenses in tissues such as the liver, intestine, muscle, and gonads to cope with oxidative stress associated with mating behaviors or territorial disputes. Additionally, the differential expression of the *sod*s genes in various tissues could be attributed to sex hormone-mediated regulatory mechanisms (Uzun and Bayır, 2023). Sex-biased genes often show elevated rates of protein sequence and gene expression divergence between species, which may be influenced by factors such as sexual selection and sexual antagonism (Grath and Parsch, 2016). These transcriptional differences can contribute to sex-specific traits and disease susceptibilities, highlighting the importance of considering sex as a biological variable in gene expression studies across tissues and species. Testosterone, for example, has been shown to influence antioxidant enzyme activity and gene expression in fish (Elsevar and Bayır, 2023). Therefore, the observed transcriptional differences may be linked to the modulatory effects of sex hormones on the *sod*s genes expression in male Tetraodon. Moreover, the *sod*s genes's role in protecting tissues from oxidative damage suggests potential functional implications of its differential expression between male and female fish. Elevated expression levels in male fish may confer greater antioxidant capacity and resilience to oxidative stress, which could be advantageous in environments characterized by fluctuating oxygen levels or exposure to environmental toxins. However, it's important to consider the limitations of the study, such as the sample size and potential confounding factors that were not accounted for. Further research, including experimental manipulation of sex hormone levels or environmental stressors, may provide deeper insights into the mechanisms underlying the observed transcriptional differences in *sods* genes expression between male and female Tetraodon individuals.

In conclusion, our findings highlight the complexity of sex-dependent regulation of antioxidant defenses in Tetraodon fish and underscore the importance of considering tissue-specific differences in gene expression when studying

oxidative stress responses in vertebrates. Further investigation into the molecular mechanisms governing sod1 gene expression in different tissues and under various physiological conditions is warranted to elucidate its functional significance in antioxidant defense and overall health of Tetraodon populations.

Bioinformatics studies of *sod1, sod2,* and *sod3* genes in Tetraodon (*Tetraodon nigroviridis*)

Organisms share genetic closeness, identities, and similarities, enabling studies conducted on one species to serve as a model for different species. Consequently, conducting in silico analysis of sod genes in Tetraodon, a model organism in this study, will provide pivotal data for molecular investigations in other fish species.

Fish possess three isoforms of the sod gene: copper-zinc SOD, which is encoded by the sod1 gene; manganese SOD, encoded by the sod2 gene; and extracellular SOD, encoded by the sod3 gene (Sheraz et al., 2023). Previous research has indicated that teleost fish typically possess duplicated copies of numerous genes, a characteristic not commonly observed in other vertebrates, which usually have single copies of these genes (Braasch and Postlethwait, 2012; Taşbozan et al., 2022). *Tetraodon nigroviridis* genome has only one copy of the sod genes (*sod1, sod2,* and *sod3*), in contrast to the zebrafish, common carp (*Cyprinus carpio*), and goldfish (*Carassius auratus*) genomes, which have two copies of sod3 (*sod3a* and *sod3b*). The loss of *sod3a* in the tetraodon genome is thought to be due to non-functionalization, which is a common outcome in the variation of duplicated genes (Glasauer and Neuhauss, 2014; Bayır and Özdemir, 2023). A search of the Ensembl database revealed single copies of *sod1* and *sod2* genes not only in tetraodon but also in many other fish species, such as zebrafish, platyfish, Amazon molly, brown trout, common carp, fugu, Nile tilapia, goldfish, and stickleback. This finding suggests that the loss of other coppy of *sod*s genes in the tetraodon genome is not unique to this species and may be a common occurrence in various fish genomes. Asymmetrical selective pressure refers to the differential selection of gene copies, which can lead to the retention of specific copies while others are lost over time. Biased gene loss, on the other hand, involves the preferential loss of certain gene copies due to factors such as gene dosage, expression levels, or functional redundancy. This process can lead to an enrichment of specific gene functions, such as developmental, signaling, or behavioral genes, in certain species or lineages.

The search results indicate that the tetraodon identity/similarity rate of Sod1, Sod2, and Sod3 sequences with their respective orthologous is higher than with their respective paralogous. This is likely due to Sod/SOD polypeptides being related to the ancestral gene, and the phylogenetic tree shows a strong evolutionary relationship between tetraodon Sods and Sods/SODs from vertebrates, suggesting that tetraodon sods are orthologs of *sod*s/*SOD*s.

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AUTHORSHIP CONTRIBUTIONS

The manuscript, produced from Büşra Kaya's master thesis, involves collaborative contributions from the authors. Büşra Kaya was responsible for the literature review, drafting, writing, conducting laboratory experiments, and managing and analyzing data. In contrast, Mehtap Bayır contributed through conceptualization, drafting, writing, reviewing, editing, and supervision. All authors have reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS APPROVAL

The research adhered to all relevant international, national, and institutional guidelines for the ethical care and use of animals.(Ankara University, Date: 30.07.2021/No: 177)

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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