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GH overexpression modifies muscle expression of anti-oxidant enzymes and increases spinal curvature of old zebrafish

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ABSTRACT

Growth hormone (GH) excess causes an increment in the metabolic rate and in reactive oxygen species generation, which accelerate the ageing process in mammals. Considering that there is no information on this subject in fish, the aim of the present study was to evaluate the excess GH effect on senescence in a zebrafish (*Danio rerio*) transgenic model. In order to reach this objective, we analyzed the phenotype of spinal curvature and expression of genes related to the anti-oxidant defense system and myogenesis in muscle of 8 and 30 months old GH-transgenic males. Gene expression analyses revealed that both superoxide dismutase isoforms were down-regulated only in 30 months old animals, while glutamate cysteine ligase was down-regulated in GH-transgenic zebrafish. Acceleration of the spinal curvature and a reduction in the expression of miogenin at both ages and MyoD in the old fish were also observed. Although neurolipofuscin accumulation was not significant in GH-transgenic zebrafish, the estimation of maximum longevity based on the von Bertalanffy growth function was significantly lower in this group. The results obtained here indicate that GH overexpression reduces the transcription of anti-oxidant defense system and myogenesis-related genes, which probably accelerates senescence in the zebrafish transgenic model used.

1. Introduction

The senescence mechanism is characterized as alterations in an organism related to the passage of chronologic time that adversely affects its vitality and functions and, more than this, increase the mortality rate of a population in a time function (Finch, 1990). In addition, senescence alters several basic processes such as reproduction, energy generation, mechanisms to avoid and efficiently repair cellular damage generated in oxidative damage and replication process, several hormonal levels and the capability to respond to stress situation (Brown-Borg and Rakoczy, 2003).

Age-related loss of muscle mass, called sarcopenia, is one of the most marked problems associated with ageing. Animal experiments have shown that older muscles are injured easily and regenerate more slowly, and that this results in impaired functional recovery (Goldspink and Harridge, 2004). It has been shown that satellite cells are responsible for the majority of post-natal skeletal muscle growth and the molecular events that occur following its activation, differentiation and fusion to form multinucleated myotubes have been well established (Holterman and Rudnicki, 2005). The differentiation and fusion program is strictly regulated by several transcription factors, being the key players the paired box transcription factors (PAX), myogenic regulatory factors (MRFs: including Myf5, Myf4, MyoD and myogenin) (Kuang and Rudnicki, 2008). Myostatin is also another important molecule in the control of muscle fibers generation and growth, which suppresses the action of the myogenic factors (Joulia-Ekaza and Cabello, 2007). It was previously stated that satellite cells have a limited capacity to divide entering a state of irreversible growth arrest after a finite number of cell divisions (Lorenzon et al., 2004).

The endocrine system plays a major role in aging of several organ systems. An age-associated decrease in the secretion of several hormones (including steroids) is observed in senescent organisms. Several endocrine mutants and/or transgenic animals have been utilized in aging studies concerning the effects of the somatotrophic axis in the aging signaling. The somatotrophic axis consists of hypothalamic hormones, GH, IGFs and downstream signaling

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molecules. GH and IGF-1 have both somatic effects stimulating the growth of tissues and metabolic effects that play a role in protein, carbohydrate and lipid metabolism (Butler and Roith, 2001). The alteration in the whole organism metabolism elicits a general induction in the metabolic rate, including several fish species (Seddiki et al., 1996; Cook et al., 2000; Deitch et al., 2006; Rosa et al., 2008). It has been demonstrated that this pathway affect processes involved in aging and longevity, and it seems that this axis plays a key role in the determination of life span in multiple species suggesting an evolutionary conservation (Brown-Borg et al., 2009). A reduction in GH secretion and IGF-1 is observed in humans, beginning in the third decade of life. The rate of GH secretion in adults fails with increasing age by approximately 14% per decade of adult life. This decline is postulated to be causative of many physical disturbances in aging organisms, including reduced strength and muscle mass (Toogood and Shalet, 1998).

Several studies have demonstrated that excessive GH plasmatic level is associated with a general reducing of life spans in mammals and other model organisms. In the same way, animals that presented a deficiency in one of the main factors in GH signaling pathway (GH, IGF-1 and membrane receptors) possesses an increased life span (Brown-Borg, 2009). This general GH effect of reducing life span is related to an enhancement of the metabolic rate, which is accompanied with an increase in the reactive oxygen species (ROS) production. Intense investigations focusing physiological alterations induced by ROS and their role in aging processes are in progress. Aerobic organisms possesses a well developed anti-oxidant defense system, that is comprised by both enzymatic and non-enzymatic molecules present in virtually all cells in order to eliminate ROS, reducing its potential toxic action on DNA, lipids and proteins (Storey, 1996). It was previously stated that 0.1% of the total oxygen consumed in aerobic process is converted in ROS (Fridovich, 2004). Thus, an augmentation in the metabolic rate leads to an overall increase in the production of these harmful molecules. In this sense, it is expected that an organism exposed to an elevation of intracellular ROS production increases their anti-oxidant defenses in order avoid deleterious effects (Morel and Barouki, 1999). However, it has been previously demonstrated that organisms exposed to elevated GH doses, that elicits higher metabolic rates, presented several of its anti-oxidant defense systems down-regulated (for a review see Brown-Borg et al. (2009)).

Recently, it was proposed the utilization of fish species for studies on aging process (Gerhard and Cheng, 2002). The widespread and genetic characterization of these vertebrates has led to many exciting discoveries in the field of developmental biology and disease (Keller and Murtha, 2004). The zebrafish *Danio rerio* offers several advantages over mammalian models for senescence studies, including the conservation of genes across vertebrates, small size, external fertilization and transparent embryos to perform mutagenesis and genetic manipulation, and the capability to maintain thousands of animals in a small place (Keller and Murtha, 2004). Zebrafish possesses a very gradual senescence pattern (Kishi et al., 2003), and a mean life span of 36–42 months, with a maximum life span of 66 months (Gerhard et al., 2002). These animals have the same mammalian pattern of ageing associated with loss of muscle mass and strength (sarcopenia), which is the main cause of the senescent phenotype of spinal curvature observed in elderly fish (Gerhard et al., 2002).

It has been previously reported that GH-transgenic zebrafish from F0104 lineage, which overexpress a piscine GH (Figueiredo et al., 2007a), possesses an increased metabolic rate and diminished muscle gene expression of the glutamate cysteine ligase catalytic subunit (GCLC) enzyme, the rate step limiting enzyme responsible for the synthesis of main non-enzymatic ROS scavenger, the tripeptide glutathione (Rosa et al., 2008). Considering the previous information, the aim of the present study is to evaluate the effects of the GH overexpression on the gene transcription profile of some anti-oxidant enzymes and myogenic differentiation factors, and on the degree of spinal curvature level in zebrafish from the F0104 GH-transgenic lineage.

In order to test the hypothesis that GH excess is accelerating senescence in transgenic zebrafish, lipofuscin and growth curve were also used as classical age markers. Lipofuscin is a "waste" product resulting from cellular metabolism that accumulates in secondary lysosomes (Terman and Brunk, 1998). This pigment is found in many tissues, but the most conspicuous accumulations are observed in post-mitotic cells, where it is not "diluted" by cellular division resulting in an accumulation with age. This age-related accumulation is the support for the use of lipofuscin for age determination. In the same sense, growth curve describes the increase in size as animal ages. One of the best known growth models is the von Bertalanffy growth function, which is anchored in a growth constant (K parameter) that is directly related with fish longevity (Pauly, 1980).

2. Material and methods

2.1. GH-transgenic zebrafish and maintenance conditions

In the present study, it was used non-transgenic (NT) and the GH-transgenic zebrafish lineage (F0104) developed by Figueiredo et al. (2007b). This lineage possesses two transgenes on the same chromosome, one of which contains the growth hormone cDNA from the marine silverside *Odonthestes argentinensis* and the other one contains the green fluorescent protein gene (GFP). Both of these transgenes are driven by the carp (*Cyprinus carpio*) β-actin promoter. Transgenic (GH) and non-transgenic (NT) sibling fish were produced by crossing hemizygous transgenic males and wild-type females. The offspring was analyzed by epifluorescence microscopy (excitation 485 nm, emission 520 nm) for transgenic fish identification through GFP expression.

Transgenic and non-transgenic zebrafish were reared until 8 and 30 months of age in a closed circulation water system composed of 25 L tanks at 28 °C, 14 h light/10 h dark photoperiod, fed with high-protein (47.5%) commercial food (5% of body weight/day). The groups were maintained separately in a 1.25 animal/L density until 3 months old. After that, fishes were raised in a proportion of one fish to 2.5 L. Each tank contained approximately 15 fish of both sexes. The growth curve data were obtained from both sexes from 3 to 6 months old zebrafish.

Two extra tanks were maintained with 8 males for 8 and 30 months for spinal curvature determination, standard length measurement and molecular analyses. At the end of the experimental period fishes were anesthetized (tricaine 0.1 mg/mL), radiographed and sacrificed in ice. All the experiments procedures done in the present study comply with current Brazilian laws.

2.2. Gene expression assays

Total RNA was extracted from muscle using TRIzol reagent (Invitrogen) ($n = 5$ per group). RNA concentrations were determined with the Qubit Fluorometer and the Quant-iT RNA BR Assay Kit (Invitrogen). RNA integrity was checked in 1% agarose gel electrophoresis. RNA was treated with RNAse free DNAse I (Applied Biosystems) following the manufacturer's instructions. Total cDNA was prepared from 2.5 μg total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

The obtained cDNA was used as template for gene amplification using specific primers designed with basis on specific gene sequences available at GenBank (www.ncbi.nlm.nih.gov). The source sequences and designed primers are described in Table 1. The

primers were designed using the Primer Express 2.0 software (Applied Biosystems).

Quantitative PCR was performed with an ABI Prism 7300 Sequence Detection System (Applied Biosystems) using SYBR-Green PCR Master Mix (Applied Biosystems). The thermocycling program was 40 cycles of 95 °C for 15 s and 60 °C for 1 min, with an initial cycle of 95 °C for 10 min. One-tenth of the cDNA was used for each Q-PCR reaction in a total volume of 20 μL. Triplicates were run for each sample. The expression levels of target genes were normalized using the expression of a housekeeping gene, elongation factor 1α (ef1α). The mRNA abundance of ef1α relative to total RNA did not exhibit any significant differences between experimental groups. No-template controls confirmed the absence of interfering non-specific amplicons such as primer–dimers. Primer sets that resulted in non-specific amplification were redesigned. The absence of non-specific amplification was also confirmed by examination of dissociation curve profiles for each sample to confirm a single peak at the expected melting temperature. The linearity of the Q-PCR assays and the absence of inhibitors in the RNA samples were confirmed using dilution series. The efficiency of each set primers was also checked.

2.3. Radiographic analysis and spinal curvature measurements

The radiographs of animals were taken according to Gerhard et al. (2002), with brief modifications. Fish ($n = 8$ per group) were radiographed in groups using a GE Senograph DMR Plus mammographic system (General Electric Medical Systems) to allow good resolution of the fish skeletal system. Images were obtained using a magnification technique (24 Kvp, 14 mAs) with a dedicated mammographic film screen system. The length of the skeleton was measured on the images obtained from scanned mammographic film screen. The measurements were done with the free software Image J (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). In order to determine the degree of spinal curvature it was measured the length (L) of the vertebral column (from the first to the last vertebra) and the straight-line distance from the first to last vertebrae (SL) (Fig. 1). The spinal curvature was calculated as L/SL. Without curvature, the length of the vertebral column is almost identical to the distance from the first to last vertebrae producing a ratio close to 1.

2.4. Neurolipofuscin analysis

The main property of lipofuscin, as far its identification is concerned, is its autofluorescence in unstained tissues. In neural tissues using an excitation of 514 nm wavelengths (green) with a barrier filter >550 nm, *in situ* lipofuscin is identified by emission maximum of 580 nm (yellow/golden). Brain samples ($n = 3$ per group) of 30 months old GH-transgenic and non-transgenic zebrafish were analyzed. Neurolipofuscin was quantified in unstained sections of brains. Serial sections (6 μm thick) containing the telencephalic ventricle were collected, in which neurolipofuscin was quantified in periventricular cells. For each section, the region with the most conspicuous neurolipofuscin accumulation was selected and an image was acquired (objective 100×; zoom 3.0×), and saved in a grayscale color pattern. For image analysis, neurolipofuscin granules appear brighter against the darker cellular background and the proportion of the image occupied by neurolipofuscin was obtained, for each section, by measuring the ratio between the area of neurolipofuscin granules and the total area of the background cells. An average of each sample (typically 15 sections by sample) was estimated and expressed as % of neurolipofuscin.

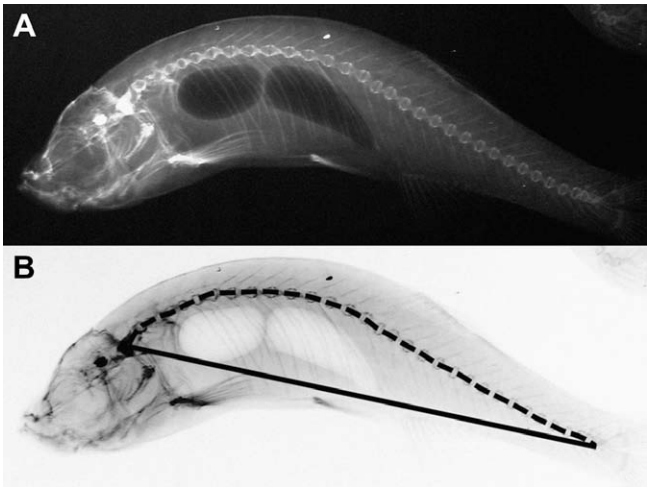

Fig. 1. Radiographic images of a 30 months old GH-transgenic zebrafish (*D. rerio*). (A) Scanned image of a mammographic film screen of the animal. (B) Negative from the same image representing the measurements performed. Solid line represents the straight-line distance from the first to the last vertebra (SL). Dashed-line represents the total length of the vertebral column (L).

2.5. Growth curve analysis

Growth curves were estimated for GH-transgenic and NT zebrafish. The von Bertalanffy growth function (VBGF) was fitted to total length (TL) data obtained from the same tank at regular age intervals during rearing (between 90 and 180 days after hatchery). VBGF is determined by the formulae $L = L_\infty[1 - e^{-K(t-t_0)}]$, which has three main parameters: K (growth constant), L_∞ (asymptotic length), and t_0 (age at length = 0). This function has been widely used to model fish growth (Moreau, 1987). Fitting was performed by minimization of least squares and significance was provided by a F-test.

The K parameter can be considered an index of theoretical longevity (Taylor, 1962; Pauly, 1980), and it has been assumed that

Table 1
Gene-specific primers used for quantitative PCR.

Gene	Function	GenBank Accession Number	Forward primer (5′–3′)	Reverse primer (5′–3′)
Catalase	H_2O_2 degradation	BC051626	TGATCTTAGCAAATGCAACACTGA	TGCAAAGGCCCCCATTTT
GPx1a	H_2O_2 degradation	BC083461	CCAAGTAAACCAGCGGCTTCT	TGATGTGCAGTGGACGGTTTAT
GCLC	Glutathione synthesis	BC068331	ACGGCATTCCCCAGGTTAG	TTTTCAACAGGTGTGGGTTTGTA
Cu, Zn-SOD	Superoxide anion dismutation	BC055516	GGAAGAGCCGGTTGAAATATTG	AGCGGGCTAAGTGCTTTCAG
Mn-SOD	Superoxide anion dismutation	BC060895	GAATGTCAGCGAGCGTTTCC	TCCCAAGTGTGGTGCAATTTATT
Myogenin	Myogenic regulatory factor	AF202639	CCTTCAGACCAGCTTTCACTGA	CCAAGGCTTGTCTAACTTGCAA
MyoD	Myogenic regulatory factor	BC114261	GGAGCGAATTTCCACAGAGACT	GTGCCCCTCCGGTACTGA
Myostatin	Negative regulation of growth rate	AF540956	TGCTTTCCGCAAGACACTGT	GAAGCGGTGCCCAGAGAGT
ef1α	Protein synthesis	L47669	CAAAATTGGAGGTATTGGAACTGTAC	TCAACAGACTTGACCTCAGTGGTT

the oldest fish typically reach 95% of the asymptotic length. Rearranging the VBGF and inserting 95% of L_{∞} as L_{max}, maximum longevity (t_{max}) can be estimated as $t_{max} \approx 3/K$.

2.6. Statistical analysis

The gene expression significances were accessed by the Relative Expression Software Tool – REST (Pfaffl et al., 2002). Data from measurement of spinal length and spinal curvature index were tested through one-way ANOVA followed by post hoc mean comparisons test (Newmann–Keuls test). Analysis assumptions (normality and variance homogeneity) were previously verified. Neurolipofuscin data were analyzed by *t* test. In all analyses, significance was assumed when $p < 0.05$.

3. Results

Concerning the anti-oxidant defense system, it was observed a reduction in the expression of some genes in muscle of GH-transgenic animals (GH) of the two analyzed ages when compared with the respective non-transgenic (NT) siblings (Fig. 2). The GCLC gene expression was down-regulated in both ages when compared with NT animals of the same age ($p < 0.05$). The 30 months old GH-transgenic animals presented also the two superoxide anion dismutase isoforms, Cu, Zn-SOD and Mn-SOD, down-regulated ($p < 0.05$). The other analyzed genes, catalase and glutathione peroxidase selenium dependent, did not present any significant alteration when compared with NT animals ($p > 0.05$).

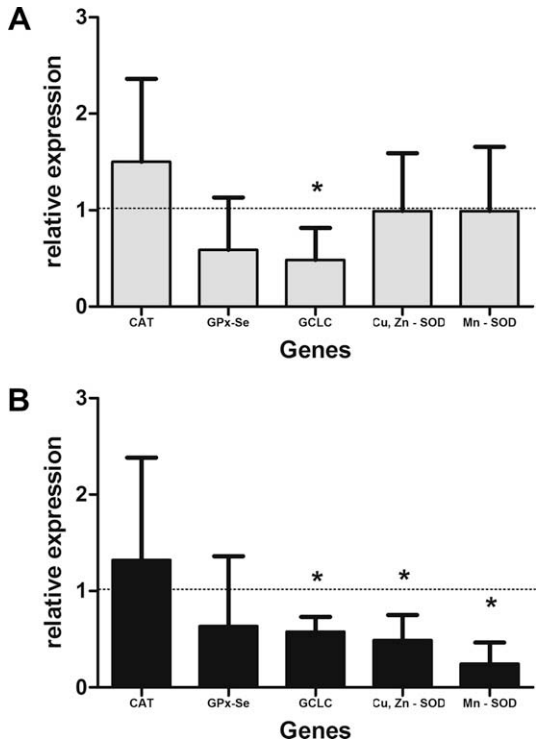

Fig. 2. Antioxidant defense system gene expression profiles. The graphs represent the relative gene expression of the 8 (A) and 30 months old (B) GH-transgenic animal related to non-transgenic siblings at the same age. The dashed-line represents the relative expression of NT animals. The gene expression was normalized by the expression of elongation factor 1α (ef1α) gene. Statistically significant differences ($p < 0.05$) in the gene expression levels between NT and GH-transgenic animals are denoted by an asterisk (*). CAT, catalase; GPx-SE, glutathione peroxidase selenium dependent; GCLC, glutamate cysteine ligase catalytic subunit; Cu, Zn-SOD, superoxide anion dismutase mitochondrial isoform; Mn-SOD, superoxide anion dismutase cytosolic isoform.

The myogenic regulatory factors presented differential expression in groups of both ages (Fig. 3). Myogenin gene was down-regulated in GH-transgenic fish of both ages ($p < 0.05$) when compared with NT. MyoD gene was down-regulated only in the 30 old GH-transgenic ($p < 0.05$). Myostatin gene did not present any difference in expression in both analyzed groups.

Concerning the standard length measurement, Fig. 4A shows that transgenic fish were lengthier than non-transgenic at the same age ($p < 0.05$), and that 30 months old fish, independently of the genotype, were lengthier than the fish of the lower age ($p < 0.05$). In addition, Fig. 4B demonstrates that GH overexpression causes no effect in the spinal curvature of 8 months old zebrafish, while in 30 months old animals it was observed a significant increase of about 5.6% in this parameter ($p < 0.05$). The older animals, independent of the genotype, presented higher curvature when compared with the younger ones ($p < 0.05$).

Regarding on neurolipofuscin, no significant difference was found between 30 months old GH-transgenic (0.096 ± 0.03%) and non-transgenic zebrafish (0.077 ± 0.01%) ($p > 0.05$, *t* test).

However, significant growth curves ($p < 0.05$) were fitted to GH-transgenic (Fig. 5A) and NT zebrafish data (Fig. 5B). The growth curve of GH-transgenic zebrafish had a $K = 1.98$/year, which resulted in an estimated $t_{max} = 1.51$ years. The same analysis for NT zebrafish indicated a $K = 0.86$/year, with an estimated $t_{max} = 3.48$ years.

4. Discussion

It has been proposed that growth hormone (GH) plays a pivotal role in the aging process (Bartke, 2006). Several animal models

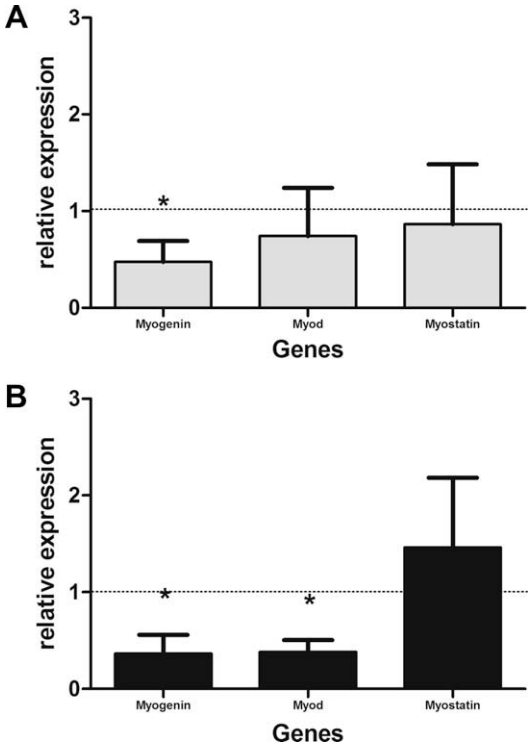

Fig. 3. Muscle-specific genes expression profiles. The graphs represent the relative gene expression of the 8 (A) and 30 months old (B) GH-transgenic animal related to non-transgenic siblings at the same age. The dashed-line represents the relative expression of NT animals. The gene expression was normalized by the expression of elongation factor 1α (ef1α) gene. Statistically significant differences ($p < 0.05$) in the gene expression levels between NT and GH-transgenic animals are denoted by an asterisk (*).

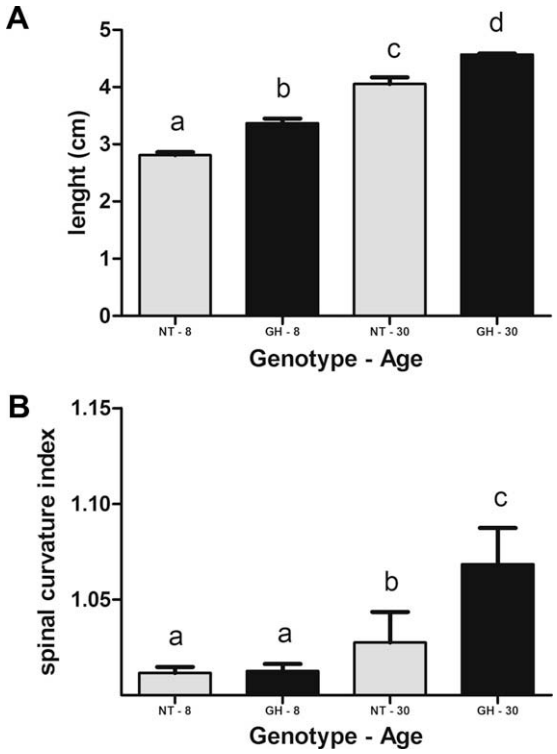

Fig. 4. Standard length measurements and spinal curvature index determinations. (A) Representation of the standard length measurements of the 8 and 30 months old NT and GH-transgenic animal. (B) Spinal curvature index determination of 8 and 30 months old NT and GH-transgenic animal. The results are presented as mean ± 1 standard error. Different letters indicates significant differences ($p < 0.05$) in the parameter between groups.

with an alteration in the GH/IGF axis have proved that this signaling pathway interfere with the maximum life span of several organisms (Kenyon, 2001; Rincon et al., 2005). On this basis, GH deficiency, GH resistance and reduced IGF-1 signaling in mice are associated with symptoms of delayed aging and markedly extend longevity (Bartke, 2006). The very plausible mechanism proposed to this extension in longevity is that reduction on the somatotrophic axis signalization leads to a reduced oxidative metabolism and reduced generation of reactive oxygen species (ROS) (Brown-Borg, 2009). ROS have been widely postulated to play a causal role in the aging process (Sohal, 1993). Indeed, the rate of ROS production together with the ability of organism to respond to the oxidative stress is strictly connected to the aging and life span (Finkel and Hollbrook, 2000). The rate of ROS production in an organism has to be counteracted by an efficient anti-oxidant defense system, capable to avoid the oxidative stress situation and generation of oxidative damage in lipids, proteins and DNA (Storey, 1996).

The reduction in oxygen consumption, body core temperature and mitochondrial ROS production, combined with increase expression and or activity of enzymes of the anti-oxidant defense system in hypopituitary and GH-resistant mice are obviously consistent with the proposed mechanisms for delayed senescence (Bartke, 2006). By the other hand, it has been demonstrated that GH overexpression or GH treatment elicits the contrary pattern of augmentation of the metabolic rate and ROS production and lowering of the expression and/or activity of multiple enzymes of the anti-oxidant defense system in mammals (Brown-Borg and Rakoczy, 2000, 2003, 2005; Hauck and Bartke, 2000; Brown-Borg et al., 2005; Donahue et al., 2006).

Considering that, at our knowledge, there is no information about the relationship between the somatotrophic axis and senescence in fish, we have analyzed the effects of the GH overexpression in some major players in the aging process in a zebrafish (*D. rerio*) transgenic model. It was previously demonstrated that animals from this lineage (F0104) possesses an augmented metabolic rate and increased ROS production in muscle (Rosa et al., 2008). The same authors had also observed none alteration in muscle activity of catalase (CAT) and superoxide dismutase (SOD), two anti-oxidant enzymes. These enzymes act in a coordinate manner, where SOD catalyzes the dismutation of the superoxide anion (O_2^-) and CAT degrades the hydrogen peroxide (H_2O_2) produced. H_2O_2 is also degraded by the enzyme glutathione peroxidase selenium dependent (GPx-Se), which utilizes the tripeptide glutathione as a co-substrate (Storey, 1996). An imbalance in the activities of these enzymes would cause oxidative stress to the organism (Fridovich, 1998).

The present study has analyzed the gene expression of CAT and two SOD isoforms (Cu, Zn-SOD and Mn-SOD). In agreement to the previous findings of Rosa et al. (2008) it was not observed any significant alterations in CAT gene expression in muscle of 8 and 30 months old GH-transgenic zebrafish. Hauck and Bartke (2000) have presented elegant findings working with hypothalamic tissue of both prolonged life span Ames dwarf mice and GH-transgenic mice with reduced life span. These authors have observed increased CAT activity in dwarf mice and lowered in GH-transgenic mice. In addition, an age-related decline in CAT activity was also observed in all experimental groups, a finding not observed in our study. However, these controversial results could be explained by the fact that the tissues analyzed in both studies are not the same. Indeed, Brown-Borg and Rakoczy (2003) have previously reported the tissue-specific GH effect. Also, it is worth to emphasize that in the present study we are analyzing the CAT gene expression and not CAT enzyme activity. Finally, the phylogenetic distance between fish and rodents could imply in different responses to GH-induced ROS increasing. These observations must be taking in account when comparing different animal models.

Considering GPx-Se gene expression, it was not observed any difference in both analyzed ages (8 and 30 months). This result is different from that observed by Brown-Borg and Rakoczy (2003) in GH-injected dwarf mice, in which a reduction in the GPx-Se content was observed in muscle, liver and kidney. Despite of ROS is enhanced in GH-transgenic zebrafish (Rosa et al., 2008), the absence of induction of GPx-Se and CAT gene expression observed in the present study suggests a reduction in the capability of hydrogen peroxide degradation, a fact previously observed in mice models (Brown-Borg and Rakoczy, 2000).

Rosa et al. (2008) had observed a reduction in the GCLC gene expression in muscle of 4 months old GH-transgenic zebrafish. In the present study it was observed the same pattern of reduction in the GCLC gene expression of the 8 and 30 months old transgenic animals. In the same way, previous reports have demonstrated that the GCLC protein levels in muscle and liver of GH deficient mice were significantly elevated in relation to the wild-type animals at all ages analyzed (Brown-Borg et al., 2005). Recently, Brown-Borg et al. (2009) have reported that the GCLC liver protein levels of GHR knockout mice are higher than the wild-type animals. However, these authors have not observed any significant differences in GCLC gene expression in this tissue comparing GHR knockout and wild-type mice (Brown-Borg et al., 2009). This enzyme is the rate step limiting enzyme in the glutathione (GSH) synthesis, the major non-enzymatic anti-oxidant in all eukaryotic cells (Griffith, 1999). This tripeptide is observed in a mM range of concentration in mammalian cells, and it is considered the major first line of defense against ROS (Iles and Liu, 2005). The expression of GCLC gene is strongly affected by hormones, cellular redox state, amino acids availability and GSH negative feedback regulation (Griffith, 1999).

Concerning the SOD genes expression, it was observed a clear reduction of both isoforms in muscle of 30 months old transgenic

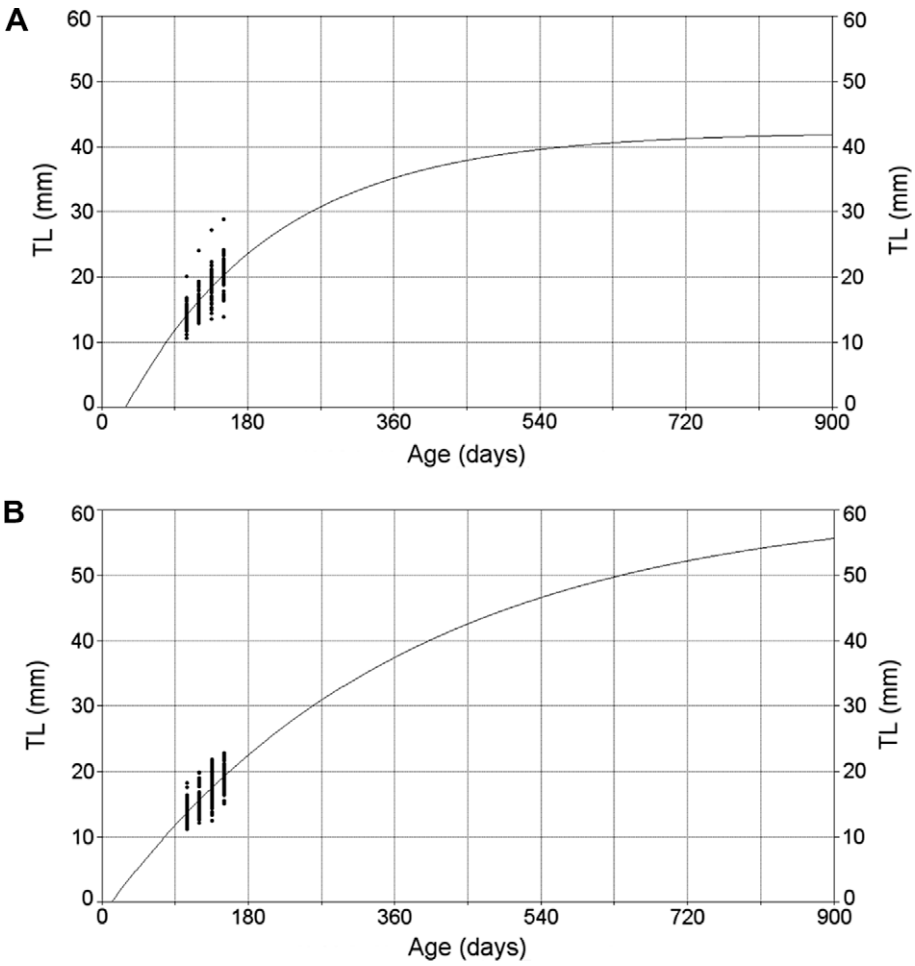

Fig. 5. Growth curves for GH-transgenic (A) and NT zebrafish (B) estimated by von Bertalanffy function of total length (TL). Dots represent the standard length of each fish ($n = 15$).

animals. The age-dependent reduction in activity of Cu, Zn-SOD was previously observed in mice overexpressing GH (Hauck and Bartke, 2000). These authors stated that, in general, Cu, Zn-SOD activities decline with age. However, these declines occurred at different chronological ages in the analyzed genotypes (Ames dwarf, GH overexpressing and wild-type animals). This decrease is accelerated in GH overexpressing and lowered in GH deficient animals. The absence of alteration in the gene expression in younger animals is in agreement with previous observations of Rosa et al. (2008) regarding SOD activity in muscle of 4 months old animals. This leads us to speculate that the reduction in both SOD isoforms gene expression is a result of the accelerating aging process. In this sense, growth hormone overexpression causes a significant reduction in the anti-oxidant capability of animals, corroborating with the general idea that GH excess decreases the capability of an organism to respond to a stressful situation.

Several senescent phenotypes have been described for fish species, including for different zebrafish strains (Genade et al., 2005). Among these phenotypes, one of the most common observed is the spinal curvature. Older zebrafish manifest various degrees of spinal curvature, as reported in other aging fish species (Gerhard et al., 2002; Gerhard and Cheng, 2002). In the present study, it was observed an increase in the standard length of GH-transgenic zebrafish when compared with the NT group in both ages. This acceleration of growth is accompanied by an augmentation in the spinal curvature index in older animals compared with the younger ones. This result is in agreement with that observed by Gerhard et al. (2002). However, the effect of GH overexpression on spinal curvature analyzed here was evident only in the older fish, which indicates a time-dependent cumulative effect of the hormone on the ageing processes. The present study has demonstrated that GH overexpression causes senescence acceleration in zebrafish, similar to that described for mice (Brown-Borg et al., 2009).

The spinal curvature phenotype is considered as a consequence of the process called sarcopenia (Gerhard and Cheng, 2002). Sarcopenia is an age-related loss of muscle mass and it is one of the major problems associated with age. This process leads to significant impairment in the ability to carry out normal daily functions (Adamo and Farrar, 2006). Animal experiments have shown that older muscles are easily injured and its regeneration occurs slowly than in youth people, and that this results in impaired functional recovery (Goldspink and Harridge, 2004).

The skeletal muscle is a post-mitotic tissue mainly composed of multinucleated myofibers and a minority of mononuclear cells, the satellite cells. The myofibers are high oxygen-consuming tissue which are privileged aging target and suffer increased oxidative damage. The satellite progenitor cells constitute a population of myogenic cells which are responsible for myofiber growth as well as muscle repair in adult tissue (Bortoli et al., 2003). The myogenic regulatory factors (MRFs) are critical for skeletal muscle determination and terminal differentiation. This family of proteins includes MyoD and its relatives Myf5, Myogenin and Mrf4 (Berkes and Tapscott, 2005). Although MyoD or Myf5 are sufficient to regulate the entire program of myogenesis in a variety of cell types, it

appears that complex signaling events in the embryo regulate the expression of many genes associated with skeletal myogenesis (Bergstrom et al., 2002). The differentiation and fusion program is strictly regulated by several transcription factors, being the key players the paired box transcription factors (PAX) and MRFs (Kuang and Rudnicki, 2008). Myostatin is another important molecule playing a role in the control of muscle fibers generation and growth by suppressing the activation of myogenic factors (Joulia-Ekaza and Cabello, 2007). After stimulation, that can be the muscle stretch, i.e., quiescent satellite cells up-regulate members of the MRF family, MyoD or Myf5. Myf5 is required for the proliferation and possible self-renewal of these cell populations, while MyoD is required for differentiation of activated myogenic precursor (Holterman and Rudnicki, 2005). Following activation, these cells divide and their progeny enter into terminal differentiation to repair. Once fusion has occurred, the nuclei of myofibers are post-mitotic and cannot reinitiate DNA synthesis (Bortoli et al., 2003). This final differentiation is regulated by myogenin (Le Grand and Rudnicki, 2007).

In the present study, it was observed a clear alteration in the pattern of gene expression of some major players in the control of muscle differentiation. The myogenin gene is down-regulated in GH-transgenic animals of both ages. The MyoD protein is down-regulated only in 30 months old animals. Although there was observed no difference in the myostatin gene expression, our results demonstrate that the GH overexpression causes an acceleration in muscle aging and, consequently, causes an impairment of new cell differentiation and repair of injured muscle fibers. It was previously stated that the satellite cells have a limited capacity to divide entering a state of irreversible growth arrest after a finite number of cell divisions, and as a result of the continuous demand on tissue regeneration, the number of satellite cells might become lower in aged muscle and thus contribute to sarcopenia (Lorenzon et al., 2004).

In addition to spinal curvature and myogenic factors analysis, we also carried out classical age markers such as lipofuscin accumulation and growth curve. Although no significant difference has been found in the accumulation of neurolipofuscin, a trend was observed in which 30 months old GH-transgenic zebrafish has a higher average accumulation of neurolipofuscin than NT group. As it has been assumed that GH-transgenic would age faster, it would be expected a significant difference between these groups. However, while apparently inconsistent at first sight, these results might be explained by selection artifacts operating in this experiment. GH-transgenic zebrafish typically survived up to 1.5 years old, and only very few were able to reach 2.5 years of age. Therefore, it is likely that these animals were alive at 2.5 years is exactly because they had a slower rate of physiological ageing (accrued by neurolipofuscin accumulation) than an average GH-transgenic animal. Late life decline mortality rates have been observed in various species (Vaupel et al., 1998). An explanation for late life declines in mortality is the selective mortality of fast-ageing individuals (Donato et al., 1979). Variability in neurolipofuscin accumulation in older chronological age groups has been discussed elsewhere (Fonseca et al., 2005), and it is likely that this variability and the selective mortality of the physiologically fast-ageing individuals affects the trajectory of neurolipofuscin accumulation. Another explanation for the low level of neurolipofuscin accumulation observed in GH-transgenic zebrafish could be the anti-oxidant properties of green fluorescent protein (GFP). Our transgenic lineage carries the GFP as a transgenesis label, and may act as a scavenger of reactive oxygen species (Bou-Abdallah et al., 2006). However, this hypothesis remains to be tested.

On the otherwise, the estimation of maximum longevity (t_{max}) based on the growth constant (K) of the VBGF was consistent with observations gained during the rearing of zebrafish. It has rarely been observed GH-transgenic zebrafish older than 18 months, and the animals which managed to attain the oldest ages (2.5 years old) had clearly slower rates of physiological ageing as it has been demonstrated by neurolipofuscin analysis. On the other hand, 4 years old NT zebrafish are very common in the tanks, and the estimated $t_{max} \approx 3.5$ years supports this observation. The K parameter can be interpreted as a "stress factor" (Pauly, 1980), being stress understood as all factors causing an increase of O_2 consumption. Therefore, although growing at a faster rate early in live than NT zebrafish, GH-transgenic zebrafish would be subject to a trade-off in which resources directed to growth would impair late life survival. Evidence of the trade-off between growth and lifespan is well-known in the gerontological literature (e.g., Metcalfe and Monaghan, 2003).

In summary, the GH overexpression in the F0104 transgenic zebrafish lineage modifies muscle expression of anti-oxidant enzymes and increases spinal curvature of old zebrafish. The mechanisms of this alteration is probably related to an increase in the metabolic rate and ROS production caused by the anabolic effects of this hormone and, as demonstrated in the present study, the down-regulation of several genes of the anti-oxidant defense system. The combination of these two factors lead to increase in the oxidative damage process that would result in accelerated appearance of senescent phenotypes such as the spinal curvature, a consequence of sarcopenia, and impaired muscle regeneration.

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