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Influence of dietary nucleotides on growth performance and stress resistance in kutum (*Rutilus kutum* Kamenskii, 1901)

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Abstract

A feeding trial has been conducted to evaluate the effects of dietary nucleotide (NT) on growth performance and stress resistance of fry kutum (*Rutilus kutum* Kamenskii, 1901). A basal diet supplemented with 0 (control), 0.3, 0.5 and 0.7 g NT kg^{-1} have been formulated for four experimental diets. After 8 weeks of feeding trial, growth parameters were evaluated and then fish has exposed to osmotic stress. To elucidate the underlying physiological mechanisms, cortisol, apoptosis and HSP70 levels were measured at selected times of 0, 2 h, 8 h and 24 h after the osmotic shock. The results revealed dietary NT feed exerts a positive effect on the growth performance of kutum fries and fish fed with 0.7 g NT kg^{-1} had the highest final weight compared with other treatments (p<0.05). Results of HSP70 showed that fish fed with NT had higher levels of HSP70 expression. Also, the levels of HSP70 at 2 h increased significantly, 3-7-fold over baseline levels (0 h) and then decreased significantly at 24 h, however HSP70 levels at 24 h was 1-3-fold over baseline levels. Apoptosis and cortisol values were affected by dietary NT levels and all NT groups showed lower stress-induced apoptosis and cortisol elevation, compared to control group.

Keywords: Heat shock protein, Apoptosis, Stress responses, Dietary nucleotide.

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Introduction

Mahisefid or kutum, *Rutilus kutum* (Kamenskii, 1901), is one of the most important bony fishes for fisheries and stocking programs in the southern Caspian Sea (Kohestan-Eskandari *et al*., 2014). Nearly 60% of bony fishes catches share in this region is this species (amounted 10,000-18,000 metric tons per year) in the Iranian coast (Sayyad Bourani, 2013). However, the kutum populations have slowly decreased in recent years. As a result, the Iranian fisheries organization (IFO) launched a restocking project since 1984 (Ebrahimi and Ouraji, 2012) to restock this valuable species, IFO annually produces and releases more than 200 million fingerlings into the rivers (approximately 1 gr). However, more recent studies have been shown a total catch of kutum have a decreasing trend (e.g. from 17,196 metric ton in 2007 to 14,835 metric ton in 2008 (Hoseinifar *et al*., 2014a; Sayyad Bourani *et al*., 2014; Hoseinifar *et al*., 2016a; Hoseinifar *et al*., 2016b).

Success of aquaculture has affected by several factors that one of the ways to restocks of kutum, is production of more resistant fingerling. One important strategy is to use of dietary supplements (Hoseinifar *et al*., 2015; Hoseinifar *et al*., 2016; Hoseinifar *et al*., 2017a; Hoseinifar *et al*., 2017b). Nucleotides (NT) are semi- or conditionally essential nutrients, but they may become essential in pathological and stress conditions that demand intense nucleic acid and protein synthesis (Carver and Walker, 1995; Palermo *et al*., 2013). Most cell types can synthesise NTs from purines and pyrimidines from de novo synthesis and salvage (Burrells *et al*., 2001a). However, NTs synthesis is a metabolically costly process requiring a significant amount of energy and time, an additional source of exogenous NTs in the diet may optimize the functions of rapidly dividing tissues, particularly in extraordinary stress conditions (such as salt water transferring, vaccination, grading, net changing, etc.), rapid growth periods, reproduction, environmental changes, combating diseases and recovery from injuries (Burrells *et al*., 2001b) . So, in practice aquaculture conditions demands on available nucleotides beyond those provided in typical aqua feeds such that an exogenous supply may result in beneficial effects (Li and Gatlin Iii, 2006). The role of NT in mediating fish acute stress responses has been demonstrated by a positive influence on plasma cortisol, growth performance, capacity for osmoregulation, hemato-immunological and biochemical parameters (Burrells *et al*., 2001b; Abedian Kenari *et al*., 2012). However, research has not been conducted on the effects of NT-supplemented diet on salinity transfer or stress responses in kutum.

Transferring to seawater or alterations in environmental salinity can generally cause aquatic organisms becoming osmotically (or ionically) stressed (Deane and Woo, 2011; AnvariFar *et al*., 2016). As a result, cellular ion regulation mechanisms can be adversely affected, which in turn can cause alterations in cellular protein

damage and subsequent induction of heat shock proteins (HSP) synthesis (Deane *et al*., 2002). HSPs are highly conserved protein that upon encountering a stressor such as osmotic shock and temperature shock, the synthesis of HSPs increases and the resultant increase and accumulation of HSPs gives the cell added protection as they help to refold, re-aggregate and restore native protein conformation (YarAhmadi *et al*., 2014a; YarAhmadi *et al*., 2014b). Results of different studies in fishes shown hyper- or hypo-osmotic stresses has been found to induce gene expression of HSPs in (e.g. Palmisano *et al*., 2000; Sakamoto *et al*., 2002). To date, there is a paucity of data relating HSP and NT in fish, although (Palermo et al., 2013) exposed sole (*Solea solea*) to NT supplementation and handling disturbance and reported that feeding of NT-supplemented diets has no significant effects on HSP70 mRNA levels after acute stress. Paradoxically, damage to cells can engage one of two opposing responses: apoptosis, a form of cell death that removes damaged cells to prevent inflammation and the induction of heat shock or stress response that prevents damage or facilitates recovery to maintain cell survival (Beere, 2004). HSPs can prevent caspase activation and induction of apoptosis (Weber and Janz, 2001; Deane *et al.*, 2006; Roy and Bhattacharya, 2006). Results of different studies demonstrated that alterations in salinity or salinity acclimation increased the number of apoptotic cells (Wendelaar Bonga and Van der Meij, 1989; Hashimoto *et al*., 1998). Although many studies have been done on apoptosis and salinity stress or HSP and salinity stress, so far there is no report relating HSP70, apoptosis and salinity in fish. In addition, little is known about the capacity NT to affect apoptosis in teleost fish.

Regarding to the economic and commercial importance of kutum in aquaculture and production more resistant of fingerlings in hatchery in order to release them in the sea for restocking purposes we exposed kutum to NT supplementation to address these goals: first, to estimate the optimum dietary NT level and to determine its effect on growth performance and feed utilization of kutum fingerlings; second, to investigate the effects of salinity transfer on physiological and biochemical characteristics and stress responses such as cortisol levels, Branchial HSP70 and apoptosis of kutum fingerlings to assess changes in osmoregulatory performance following salinity transfer.

Materials and methods

Animals and rearing conditions

Healthy fries of kutum were obtained from Breeding and Cultivation Center of Shahid-Rajaei (Sari, Iran) in April 2016 and were transported to the rearing laboratory (Ghaemshahr, Iran). They were acclimated to laboratory conditions for 2 weeks in three 1000-L tanks and fed on the basal experimental diet without dietary NTs (2% body weight) (Burrells *et al*., 2001b; Choudhary *et al*., 2012). At the end of

the acclimation period, fish with an average weight of approximately 0.05 g were randomly selected and stocked in twelve 200-L fiberglass tanks (triplicate groups per dietary treatment according to four test diets) at a density of 250 fish per tank. Fish were reared at 19±1 °C with a 16:8 h light/dark photoperiod for 8 weeks. Water pH was maintained between 7.2 and 8, and dissolved oxygen between 7.5 and 8.5 mg L^{-1}. In each tank, 75% of water volume was renewed twice daily with freshwater. The experiment was carried out in triplicate.

Test diet

In the present study, the basal diet (Table 1) contained all known nutrient requirements of kutum with 13% lipid and 42% protein (Ebrahimi *et al*., 2012). The experimental diet was supplemented with AUGIC (15% free NTs/NTs, ICC. Indl. Com. Exp. e Imp. Ltda., Sao Paulo/SP, Brazil) to provide 0, 0.3, 0.5 and 0.7 g of mixed NT kg^{-1} diet to formulate four experimental diets, and each diet was randomly allocated to triplicate groups and the first treatment was considered as the control. After 8 weeks of feeding trial, growth parameters were evaluated. The proximate analysis of experimental diet was performed according to the procedures of the Association of Official Analytical Chemists (AOAC, 2000). Fish were fed by hand to apparent satiation (visual observation of first feed refusal) five times per day at h: 8, 11, 14, 16 and 18. At the end of work growth parameters calculated and then two series experiments were conducted.

Table 1: Ingredients composition of test diets fed by kutum fries.

	Control diet	0.3%	0.5%	0.7%
Ingredient (% dry weight)				
Fish meal[1]	420	420	420	420
Corn meal	80	80	80	80
Soybean meal	100	100	100	100
Wheat gluten	120	120	120	120
Wheat flour	160	160	160	160
Soybean oil	40	40	40	40
Fish oil	40	40	40	40
Mineral premix[2]	20	20	20	20
Vitamin premix[3]	20	20	20	20
NT[4]	0	0.3	0.5	0.7

[1] Pars kelika, Mirood, Iran.
[2] Contained (g kg^{-1} mix): MgS04.2H20, 127.5; KCl, 50.0; NaCl, 60.0; CaHPO4.2H2O, 727.8; FeSO4.7H2O, 25.0; ZnSO4.7H2O, 5.5; CuSO.5H2O, 0.785; MnSO4.4H2O, 2.54; CoSO4.4H2O, 0.478; Ca (IO3)2.6H2O, 0.295; CrCl3.6H2O, 0.128.
[3] Vitamin premix contained the following vitamins (each kg^{-1} diet): vitamin A, 10 000 IU; vitamin D3, 2000 IU; vitamin E, 100 mg; vitamin K, 20 mg; vitamin B1, 400 mg; vitamin B2, 40 mg; vitamin B6, 20 mg; vitamin B12, 0.04 mg; biotin, 0.2 mg; choline chloride, 1200 mg; folic acid, 10 mg; inositol, 200 mg; niacin, 200 mg; pantothenic calcium, 100 mg.
[4] ICC. Indl. Com. Exp. e Imp. Ltda., Sao Paulo/SP, Brazil.

Stress challenge

At the end of growth trial, fish were then starved for 24 h. 50 fish from each experimental group were arbitrarily and indiscriminately selected for the stress challenge. The fish were directly and

suddenly transferred from the holding salinity (freshwater (FW), 21 °C) to the experimental salinity (15 ppt, 21 °C) into a new set of triplicate tanks (Soleimani *et al*., 2012; Hoseinifar *et al*., 2014b). A total of 8 fish per treatment group were then sampled at time-zero (prior to salinity transfer), 2, 8 and 24 h following salinity transfer. The salinity selected in this trial (15 ppt) was based on the environmental salinities which kutum encounters in the Caspian Sea. Water supplied from the Caspian Sea and sea salt was used to regulate the salinity level. Tanks were covered with net screens to prevent fish from jumping out, and continuous aeration was provided to maintain dissolved oxygen near saturation levels.

Sampling Protocol

Fish were quickly removed from the treatment tanks at each time interval, and the fish were anesthetized with 200 mg L^{-1} clove solution, gills were excised immediately, subsequently whole body of fries frozen in liquid nitrogen, and stored at −80 °C for later analysis.

Growth performance parameters

The growth performance parameters including percentage weight gain (WG%), specific growth rate (SGR), feed conversion ratio (FCR), condition factor index (CF), protein efficiency ratio (PER), daily growth rate (DGR) and survival percentage were calculated as follows:

WG (%)=(final weight–initial weight)/initial weight

FCR=total feed intake (g)/weight gain (g)

CF=(Fish weight (g)/ Fish length $(cm)^3$) ×100

SGR (% day^{-1}) =((ln final weight–ln initial weight)/time (days))×100

PER=weight gain (g)/total protein intake (g)

DGR=(weight gain (g)/time (days))×100

Survival (%)=(final number of fish/initial number of fish)×100

HSP70 expression

RNA extraction steps from gills were done using acid guanidinium thiocyanate-phenolchloroform method modified according to Awad *et al*.(2011) that have earlier been used by Kolangi Miandare *et al*. (2016). cDNA synthesis was carried on according to the Fermentas protocol (Fermentas, France). The qPCR primers for HSP70 and reference genes were designed based on the conserved regions extracted from DNA sequences in GenBank and the genomic contigs of the NCBI database. qPCR primers were designed for each gene using PRIMER3.0 software program (available at http://wwwgenome.wi.mit.edu) (Table 2). The qPCR efficiency was also taken into account for selection of the best qPCR primer pair with specific and correct size. β actine gene was used as reference gene for standardization of expression levels. Real-time PCR analysis was carried out using an iCycler (Bio-Rad) with Fermentas Maxima SYBR Green qPCR Master Mix (2×) (Fermentas) and all primers at (100 nM) after protocol

suggested by Kolangi Miandare *et al*. (2016). The PCR efficiency was calculated using the following equation: $E\%=10^{(1/Slope)-1}\times100$ (Radonic *et al*., 2004). The change in relative mRNA expression of HSP70 was assessed through the standard curve method (Larionov *et al*., 2005). The obtained data were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad).

Table 2: Primers used for the real-time PCR assay of gene expression.

Gene	Accession number	qPCR primers, forward/reverse	Amplicon	Efficiency (%)
hsp70kut	KT380686.1	AGAGAATGGTGCAGGACGC CTGATGGTGGAGTTGCATTTCTC	180	98
BetaKut	DQ061948.1	CCCTGCATGGATGTGTGGAT GGGTGACACCATCACCAGAG	189	96

Apoptosis assay

The procedure for apoptosis assaying used from modified Annexin V/PI staining protocol (Rieger *et al*., 2011). Apoptosis evaluated by eBioscience™ Annexin V Apoptosis Detection Kit FITC (eBioscience, Inc., an Affymetrix company, Carlsbad, CA 92008 USA) following the manufacturer's instructions.

Cortisol extraction and ELISA

The method for cortisol extraction used from whole-body cortisol extraction procedure described by Guest et al., 2016. In this method cortisol assayed using cortisol ELISA Kit (IBL-International, RE52061, Hamburg, Germany) following the manufacturer's instructions. Absorbance at 450 nm was read in ELISA plate reader (Multiskan Ascent Microplate Photometer, Thermo Scientific).

Statistical analyses

Data are presented as means±SE and analyzed statistically by one-way and two-way analysis of variance (ANOVA) with independent variables being treatment and sampling time. All data met the assumptions of normality (Kolmogorov–Smirnov test) and homogeneity of variance (Leven test). When a significant difference was identified among groups ($p<0.05$), the Duncan post-hoc analysis was applied to compare means. All statistical analyses were conducted using SPSS statistical package version 17.0 (SPSS Inc., Chicago, IL, USA). For apoptosis data FloMax (Software for Cytometry, ver. 2.3, Partec GmbH, Münster, Germany) was used to determine the significance between control and experimental groups by paired Student's T-test. Probability level of $p<0.05$ was considered significant.

Results

Growth measurements

There were significant differences ($p<0.05$) in survival rate during the 8-week feeding trial among the four treatment groups and fish fed with NT diet had significantly lower mortality rate than those of fish fed the NT-free control diet (Table 3). Fish fed diets with 0.7 g NT kg^{-1} had the highest final weight, WG, SGR and DGR, and lower in diet with 0.3 g NT kg^{-1} after 8 weeks

of feeding. FCR had no significant differences (p>0.05) between all treatments. CF was better when the fish were fed the diet with 0.3 g NT kg^{-1}, but it had no significant differences (p>0.05) between treatments. Also, fish fed diet with 0.3–0.5 g NT kg^{-1} had higher PER than other groups after 8 weeks of feeding, but it had no significant differences (p>0.05) between dietary treatments.

Table 3: Growth performance of kutum fry fed dietary nucleotide at the end of 8th weeks of feeding trial.

	Control (0)	**3 g NT kg^{-1}**	**5 g NT kg^{-1}**	**7 g NT kg^{-1}**
Initial weight (g)	0.05±0.007[a]	0.05±0.003[a]	0.05±0.005[a]	0.05±0.009[a]
Final weight (g)	0.27±0.02[a]	0.26±0.02[a]	0.28±0.01[a]	0.33±0.01[b]
WG	453.33±41.31[a]	430.00±46.90[a]	473.33±30.11[a]	576.66±34.44[b]
FCR	1.96±0.08[a]	1.95±0.09[a]	1.96±0.06[a]	1.96±0.07[a]
CF	1.45±0.03[a]	1.53±0.07[a]	1.48±0.03[a]	1.48±0.10[a]
SGR	3.48±0.15[a]	3.39±0.19[a]	3.56±0.11[a]	3.90±0.10[b]
PER	1.21±0.01[a]	1.22±0.01[a]	1.22±0.01[a]	1.19±0.01[a]
DGR	0.46±0.04[a]	0.43±0.04[a]	0.48±0.03[a]	0.58±0.03[b]
Mortality rate (rearing period)	111±41[d]	55±17[c]	48±11[b]	46±13[a]
Mortality rate (after salinity stress)	2±1[b]	0.33±0.57[a]	0.33±0.57[a]	0.33±0.57[a]

Values are mean±S.E. of three replicate groups. Mean values with different superscripts are significantly different from each other. (Significance level is defined as p<0.05).
WG, weight gain; FCR, feed conversion ratio; CF, condition factor index; SGR, specific growth rate; PER, protein efficiency ratio; DGR, daily growth rate.

HSP70 expression

In all treatments one fish died immediately following salinity shock. The effects of dietary NT supplementation on the expression of gill HSP70 of kutum fries are shown in Fig. 1. Fish transferred to SW, HSP70 levels at 2 h increased significantly, 3-7-fold over baseline levels (0 h) and then decreased significantly at 24 h, however HSP70 levels at 24 h was 1-3-fold over baseline levels.

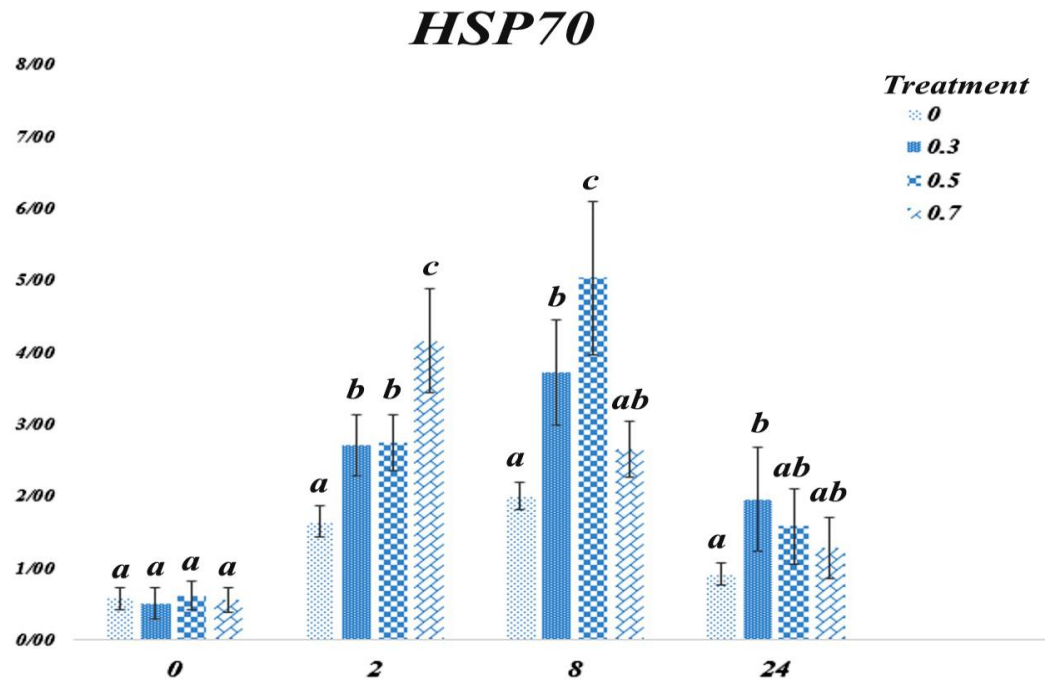

Figure 1: Changes in gill HSP70 expression after salinity shock (transfer from FW to 15 ppt seawater) in different time.

Apoptosis

The effects of salinity shock on the gill apoptosis of kutum fries that fed with different levels of dietary NT supplementation are shown in Fig. 2. Salinity shock induced apoptosis in bronchial of kutum fries and all NT groups showed lower stress-induced apoptosis, compared to that of the control group (0 g NT kg^{-1}). The populations of apoptotic, necrotic and alive cells were significantly affected by dietary NT levels (p<0.05). Prior to stress (time 0) the majority of cells were viable and non-apoptotic (71-91%) and the populations of live cells were normal (Fig. 2, down- left). However, with passing time at 8h, there was a decrease in the Annexin V–PI– population and an increase in cells undergoing early apoptosis (Annexin V+PI–) as at 24 h the populations of live cells reached to 33-51%. Prior to stress the minority of cells were apoptotic (0.81-18%). However, with passing time at 8h there was an increased in the Annexin V+PI– population (33-56%) and then at 24 h reached to peak (38-62%) and were stable (Fig. 2, down- right). Also, a slight increase in the Annexin V+PI+ population was observed at higher doses of NT (Fig. 2, up- right) indicating dead or necrotic cells in the late apoptotic/dead cell quadrants (0 h, 1-8%; 24 h, 1-10%).

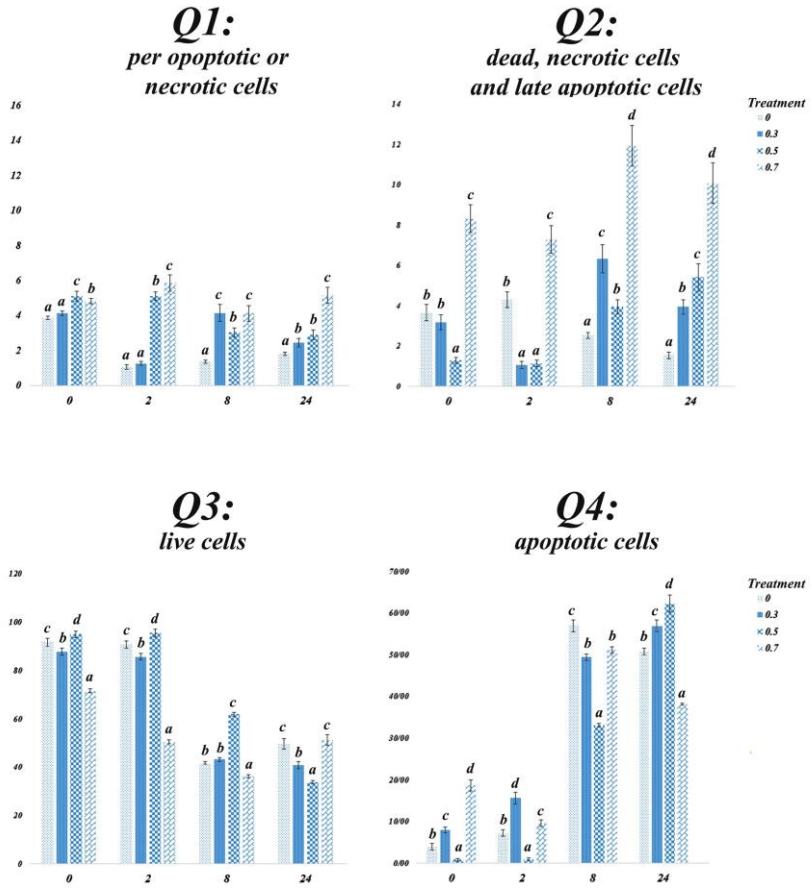

Figure 2: Changes in gill apoptosis in the kutum fries after salinity shock (transfer from FW to 15 ppt seawater) in different time. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. The lower right quadrants (Q4) are cells that stain positive for Annexin V and negative for PI (Annexin V+PI–) represent the apoptotic cells/cells undergoing apoptosis or early apoptotic cells, demonstrating cytoplasmic membrane integrity. The lower left quadrants of each panel (Q3) are cells that exclude PI and are negative for Annexin V binding (Annexin V–PI–) show live cells/ viable and non- undergoing apoptotic cells. The upper right quadrants (Q2) are cells that stain positive for both Annexin V and PI (Annexin V+PI+) contain the dead, necrotic cells/ undergoing necrosis and late apoptotic/ end stage of apoptosis cells. The upper left quadrants (Q1), are cells that stain positive for PI and negative for Annexin V binding (Annexin V–PI+) show per opoptotic or necrotic cells.

Whole-body cortisol

The effects of dietary NT supplementation on whole-body cortisol are presented in Fig. 3. Prior to stress (time 0) whole-body cortisol value in normal and NT fish were not statistically different (p>0.05). Cortisol value were significantly affected by dietary NT levels (p<0.05) and all NT groups showed lower stress-induced cortisol elevation, compared to that of the control group. Also, plasma cortisol level reached a significant peak 2 h after the salinity stress and this value decreased significantly after 8 h, returning to pre-stress levels by 24 h (p>0.05). Fish fed the 0 g NT kg^{-1} diets (2 h) had the highest whole-body cortisol (41.03±5.51). Significantly

lower whole-body cortisol was found in the diets with 0.7 g NT kg^{-1} diets (24 h).

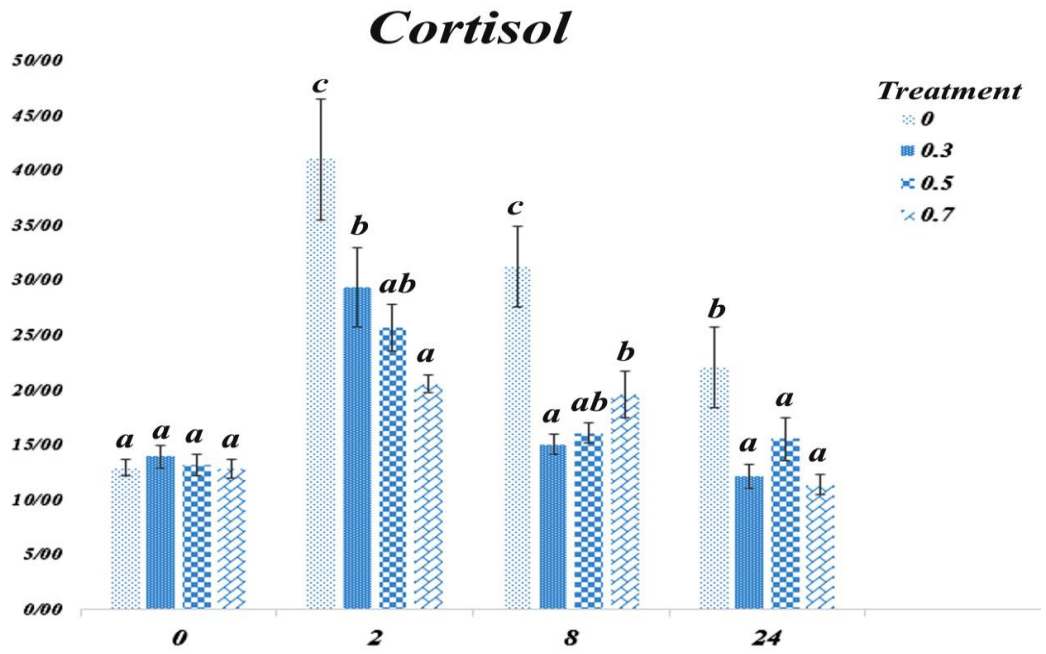

Figure 3: Changes in whole-body cortisol after salinity shock (transfer from FW to 15 ppt seawater) in different time.

Discussion

The present study showed that dietary supplementation of 0.7 g NT kg^{-1} exerts a positive effect on the growth performance of kutum fries compared with other treatments. Also, the administration of dietary NTs has been reported to enhance the growth performance at level of 1 g kg^{-1} diet in juvenile Nile tilapia (Barros *et al*., 2013), 1.5 g kg^{-1} diet in juvenile of grouper (Lin *et al*., 2009), 1.5-2 g kg^{-1} diet in fingerling of rainbow trout (Tahmasebi-Kohyani *et al*., 2012), 2 g kg^{-1} diet in Atlantic salmon *Salmo salar* (Burrells *et al*., 2001b) and in juvenile of red drum (Li *et al*., 2007), 2.5 g kg^{-1} diet in Caspian brown trout fingerlings (Abedian Kenari *et al*., 2012) and 10 g kg^{-1} diet in juvenile Nile tilapia (Anguiano, 2011). However, the growth-promoting effect of NTs has not been observed in juvenile rainbow trout (Leonardi *et al*., 2003), juvenile red drum (Li *et al*., 2005), juvenile rohu (Choudhurya *et al*., 2005), channel catfish (Welker *et al*., 2011) and juvenile hybrid tilapia (Shiau *et al*., 2015). To date, there is no exact explanation on how NTs work to enhance growth rate (Burrells *et al*., 2001b; Li *et al*., 2007b). Borda *et al*. (2003) presumed that an exogenous supply of NTs may promote growth of fish and crustaceans in early stages to meet their high rate of cell replication. However, the mechanism of growth-promotion by dietary NTs remains to be identified in fish. Some studies sustained a chemo-attractive effects of exogenous NTs, associated with the presence of some substances as adenosine monophosphate, inosine monophosphate, uridine monophosphate and adenosine diphosphate (Li and Gatlin Iii, 2006;

Barros *et al.*, 2013). Carver and Walker (1995) hypothesized that addition of NTs, especially flavor-enhancing NTs such as IMP and GMP, to food may increase the level of intake, which may increase somatic growth. However, depending on the NT mixture composition, fish life stage, different species, feeding period, exogenous NTs may have marginal or negative effect on growth (Choudhurya *et al*., 2005; Li and Gatlin Iii, 2006; Li *et al.*, 2007). In this survey, growth-stimulatory effect of NT mixture at level 0.7 g NT kg^{-1} could be due to fish stage, as larvae may have higher demand for NT due to high rate of cell replication (Barros *et al*., 2013).

Alterations in environmental salinity can generally cause stress in fish and salinity adaptation of fish is a well-known trigger of apoptotic mechanisms, especially in chloride (or mitochondria-rich) cells of the gills (e.g. Wendelaar Bonga and Van der Meij, 1989; Iger *et al*., 1994; Bury *et al*., 1998; Rojo and Gonzalez, 1999), epidermal components of the skin (Hashimoto *et al.*, 1998) and the gastrointestinal tract (Takahashi *et al*., 2006). On the other hand alterations in ambient temperature can result in a number of physiological changes including apoptosis. Under warm water temperatures, characteristic features of apoptosis such as caspase activation and DNA fragmentation have been recorded for a long time (Ito *et al*., 2008).

In present study, gill HSP levels were significantly affected by dietary NT levels (p<0.05) and groups that received higher level of NT, shown more expression of HSP70 in gills (Fig. 1). Results shown fish, were significantly elevated relative to baseline levels at 2h and reached a significant peak at 8 h after the salinity stress and this value decreased significantly after 24 h (p>0.05). Wu *et al*. (2012) demonstrated when cells were exposed to cold stress; the over-expression of HSP could be immediately induced to recover cells from stress and guard cells from subsequent insults. Palermo *et al.* (2013) evaluated the modulation of acute stress response by dietary NT in sole and demonstrated that solea HSP70 brain mRNA expression is not affected neither by handling stress procedure nor by diets. Although, environmental stressors such as alterations in environmental salinity, disease and chemical exposure are known to alter HSP expression (reviewed by Lindquist, 1986; Georgopoulos and Welch, 1993; Deane and Woo, 2011), however there are some data that shown physical handling stress caused an increase in circulating cortisol levels, did not cause an increase in HSP70 levels (Palmisano *et al*., 2000). So, it seems that different results in this study with Palermo *et al*. (2013) may be due to different type of stressors. The potential influence of the dietary NTs on HSP expression in fish is very limited. Based on current knowledge, it seems that the effect of dietary NTs on HSP expression is relate to endocrine stress response such cortisol (Palermo *et al.*, 2013). Results of different studies suggesting that dietary NT modulates stress resistance mechanisms in fish

(Abedian Kenari *et al*., 2012). Since exposure to stressors can increase demands on available NT, exogenous NT supplement may ameliorate some of the physiological responses of fish (Burrells *et al*., 2001b); and this time dietary NT could be effective in reducing the inhibitory effects of high cortisol levels on the immune system of fish exposed to stressors (Palermo *et al*., 2013). in the stress response of fish, cortisol as a key hormone play a major role (Wendelaar Bonga and Van der Meij, 1989) possibly by increasing gluconeogenic capacity and amino acids mobilization/catabolism (Mommsen *et al*., 1999). Deane *et al*. (2006) stated cortisol can directly regulate HSP70 expression in fish fibroblasts and macrophages. In addition, it is not demonstrated if exogenous NTs are involved in stress pathway modulation (Yousefi *et al*., 2012). However, more researches are needed to expand knowledge on mechanisms by which dietary NTs exert their effects HSP expression.

NTs and their derivatives can effect apoptosis. In this study the populations of apoptotic cells were significantly affected by dietary NT levels, so all NT groups showed lower stress-induced apoptosis (by salinity shock) in bronchial of Kutum fries, compared to that of the control group. The information obtained from this study correlated with Salinas *et al*. (2007), as expected. Salinas *et al.* (2007) investigated whether adenosine and other ATP derived molecules like N6-cyclohexyladenosine regulate apoptosis in leukocyte cultures from gilthead sea bream and found adenosine and N6-cyclohexyladenosine did not induce but decreased apoptosis of sea bream (*Sparus aurata*) head kidney leukocytes, in particular lymphocytes, but not phagocytes, after 24 h in culture. However, underlying mechanisms have remained unclear to date in fish. One of the possible mechanisms by which NTs beneficially influence apoptosis is partially related to cell cycle. Wang and Ren (2006) have reported adenosine regulates the cell cycle since it is capable of inhibiting apoptosis in cancer cell lines and in rat primary astrocyte cultures. In addition, nothing is known with respect to the effects of purine NTs on apoptosis of fish cells (Salinas *et al.*, 2007). However, it is not demonstrated how exogenous NTs are involved in cell cycle modulation. Another possible mechanism by which NTs affected apoptosis may be related to cortisol. Takahashi *et al*. (2006) reported during SW acclimation, intestinal apoptosis was controlled by cortisol, whereas cell proliferation during FW acclimation may involve both PRL and cortisol. As reported for many cell types (Frankfurt and Rosen, 2004), the induction of apoptosis by cortisol is possibly mediated trough glucocorticoid receptors. However, the third possible mechanisms may be related to anti-apoptotic role of HSP. Deane *et al*. (2006) stated cortisol can be anti- or pro- apoptotic via HSP70 induction or suppression respectively and increased HSP70 (achieved by treatment of cells with azetidine) could be protective possibly through suppression of

apoptosis. Taken together, further studies are required to clarify modulatory effects of NTs on apoptosis in fish.

Published researches suggest that dietary NT increases the resistance of fish to a variety of stressors (Li and Gatlin Iii, 2006). In our study, NT produced a significant reduction in measured stress response in kutum fires after exposure to salinity stress. The acute stress made plasma cortisol of fish fed with dietary NT and control group reached their maximum levels at 2 h and returned to pre-stress levels within 8 h, even reached significantly lower than the control group (at 0 h) (Fig. 3). Recently, Abedian Kenari *et al.* (2012) conducted a feeding trial to evaluate the effects of dietary NT, on the growth, haemato-immunological and serum biochemical parameters and stress responses of Caspian brown trout fingerlings and stated after confinement acute stress plasma cortisol of fish fed with dietary NTs and control group reached their maximum levels at 3 h and returned to pre-stress levels within 8 h. Leonardi *et al.* (2003) reported dietary NT in trout infected with the IPNV, reduce the levels of plasma cortisol, suggesting that this diet is able to decrease stress levels during an IPNV infection event and possibly enhancing defense mechanisms. Tahmasebi-Kohyani *et al.* (2012) demonstrated plasma cortisol concentrations of fish fed on 0.2% diet were significantly lower than fish fed the control diet at all post-stress time intervals (handling and crowding stress). Welker *et al.* (2011) found cortisol values post stress (low-water conditions and diseases challenge) were significantly lower in catfish fed the 9 g kg^{-1} and 27 g kg^{-1} NT diets compared with the control and 1 g kg^{-1} diets. However this phenomenon failed by studies in juvenile red drum (Li *et al.*, 2005). These researchers suggested that the extreme variation in individual fish may have prevented a NT-related reduction in the stress response. It seems one of the possible mechanisms by which NTs beneficially influence stress responses is partially related to inhibitory effects of stress-induced cortisol release (Li and Gatlin Iii, 2006). Often, plasma cortisol levels rise at the beginning of a chronic stress situation and decrease to initial values in a few days (Montero *et al*., 1999), showing adaptation of the fish to the new conditions.

Based on the results seems there is a significant negative relationship between expression of HSP70 and apoptosis in kutum fry gills. Results shown, with increase to cortisol concentration levels, HSP70 expression increased and with decrease that, expression of HSP70 in gills decreased. Also, the induction of HSP70 synthesis was also found to protect branchial cells against apoptosis. For example in time 2 h, when HSP70 levels increased, populations of apoptotic cells decreased while in time 8 and 24 when the trend of HSP70 is descent, the trend of apoptotic cells were ascent. So, we propose that the decrease in branchial HSP70 expression in response to salinity transfer may be causally related to the increase in gills cell apoptosis

and cortisol levels. In support of this conjecture a study on juvenile channel catfish (*Ictalurus punctatus*) has shown polyaromatic hydrocarbons exposure increased apoptosis and decreased HSP70 expression in juvenile catfish ovaries and stated this two phenomenons may be causally related together (Weber and Janz, 2001). Also reported onset of apoptosis was found to be prevented via hormonal induction of HSP70 in sea bream blood (Deane and Woo, 2005). To date, there are only a few studies that have investigated the potential role of cortisol on HSP70 and apoptosis. It was previously reported that rainbow trout hepatocytes exposed to cortisol had an attenuated HSP70 response upon acute heat shock (Boone and Vijayan, 2002) or a direct effect on the induction of a constitutive HSP70 (Vijayan *et al*., 2003). Similarly, cortisol induced apoptosis in the chloride cells of tilapia under stress conditions (Bury *et al*., 1998). Deane *et al.* (2006) demonstrated in silver sea bream fibroblast cell line (SSF), cortisol increased HSP70 and protected against camptothecin induced apoptosis (anti-apoptotic) whereas in primary culture of silver sea bream macrophages (SSM) cortisol decreased HSP70 and promoted apoptosis (pro-apoptotic). In contrast Celi *et al*. (2012) stated cortisol intra-peritoneal injection and in vitro treatment of head kidney cells induced HSP70 and HSP90 but caused no apoptotic effects in the cells. Taken together for better understanding of this conjecture, more research is needs to expand this relationship.

As previously noted, the proper size for kutum releasing in Caspian sea (13 ppt) is 2.5-3 g (Sayyad Bourani *et al*., 2014). Results of present study showed kutum fries with weighting 0.5 g that fed with NT can tolerance 15 ppt SW and survive for 24 h. Although there were no differences in timing of mortality and mortality rate between treatments. However, results of mortality shows fries at 0.5 g can survive and tolerance the osmotic shock. In conclusion, the present study showed that dietary NT supplementation exerts a positive effect on the growth performance of kutum fries. These results provide good correlative evidence of the involvement of branchial HSP70 in the osmotic tolerance. Dietary NTs in kutum challenged with the salinity, reduce the levels of plasma cortisol (NT groups, 2 h), suggesting that this diet is able to decrease stress levels during transfer to salinity.

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