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CONSECUTIVE LIVER EXPRESSION LEVELS OF CAT, Cu-Zn-SOD1, HSP70, AND GSTM1 GENES IN HEAT STRESSED MALE RATS TREATED WITH THYMOQUINONE

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ABSTRACT

The hepatoprotective impact of Thymoquinone (TQ), under chronic heat stress, was evaluated in male rats by examining the levels of liver CAT, Cu-Zn-SOD1, HSP70 and GSTM1 genes expression. Two hundred and forty male rates were equally assigned to four groups. The animals were daily administered, for 6 weeks with, distilled water under normal ambient temperature (21±1 ºC) (control), distilled water under high ambient temperature (35±1 ºC) (heat stressed; HS group), TQ suspension (50 mg/kg, bw) under high ambient temperature (35±1 ºC) (heat stressed treated with TQ; HSTQ group), and TQ suspension (50 mg/kg, bw) under normal ambient temperature (21±1 ºC) (positive control; TQ group). At the end of each week, ten males from each group have been anesthetized by single injection of thiopental (100 mg/ kg, i.p.), sacrificed, and samples from liver tissues were obtained for assessment of liver genes profile. In HS and HSTQ groups, all studied genes elevated than control level among the experimental periods but it down-regulated in HS group during the progress of heat stress, except SOD1 gene which continued in its elevation. Liver GSH gene expression levels of the three treated groups increased than control, particularly in HSTQ group. In comparison with control, HS and TQ groups, liver HSP70 gene expression was significantly higher in HSTQ group, at the last four weeks of experiment. In conclusion, TQ could be more potent in response to stress, as well as its protective and curative impact of hepatic oxidative damage under chronic heat stress.

Key words: thymoquinone, heat stress, antioxidants, oxidative stress

INTRODUCTION

Oxidative stress results from expanded creation of free radicals and reactive oxygen species, and a reduction in antioxidant combatting reported that oxidation is crucial to almost all cells in the body to give vitality to imperative capacities. Around 95 to 98% of the oxygen devoured is lessened to water during metabolism, yet the remaining might be changed over to reactive oxygen species, that may harm the DNA and causes degenerative alterations (Trevisan et al., 2001; Williams et al., 2002). It is well known that increasing ambient temperature, as recorded in the last years, is a strong oxidative stress on living organisms. Therefore it is powerfully needed to combat the morbidity and mortality associated with heat stress.

It has been shown that heat stress causes protein unfolding by chaperons, such as HSP70 which has a significant role as cell thermotolerant (King et al., 2002; Beckham et al., 2004; Jan et al., 2015), where its levels have already been associated with heat stress in humans (Kregel, 2002; Marini et al., 2007, Tuttle et al., 2015), rats (Arnaud et al., 2002) and cattle (Gaughan et al., 2013). Superoxide dismutase-1 also known as Cu-Zn superoxide dismutase gene is responsible for encoding SOD1 enzyme in liver (Milani et al., 2011). Superoxide dismutase (SOD) is currently known to facillitate the dismutation of superoxide to hydrogen peroxide and oxygen (McCord and Fridovich, 1969), where it has been mentioned that the significant resistance in detoxification of superoxide anion and hydrogen peroxide, are superoxide dismutase (SOD), catalase and glutathione peroxidase (McCord and Fridovich, 1969; Chance et al., 1979). Catalase is a heme-containing catalyst that influenced in the dismutation of hydrogen peroxide into water and oxygen. In peroxisomes catalase deals with the cytosolic and mitochondrial peroxides produced among urate oxidation (Oshino and Chance, 1977). Mitochondrial SOD promptly changes over the main part of mitochondrial superoxide particles to H_2O_2. Along these lines, SOD and catalase shields the cell from the harm because of the optional era of exceptionally reactive hydroxyl group from superoxide particle to H_2O_2 (Miyazaki et al., 1991). Glutathione is a simple protein molecule that is produced naturally in the body. It is composed of cysteine, glycine and glutamine. Its activity is related to its content of sulfur (SH) chemical groups. Normally glutathione is recycled inside the body, but in case of strong oxidative stress such as heat

stress when the toxic load becomes so great, the recycling of glutathione will impaired. The main genes that involved in producing enzymes that allow the body to create and recycle glutathione in the body are GSTM1 and GSTP1. From which, GSTM1 is the most important one (Nuttall et al., 1998).

Thymoquinone, an active constituent of *Nigella sativa* seed (Salem, 2005), has been engaged in the amelioration of lipid peroxidation (Zaoui*et al*., 2002; Kalus*et al.,* 2003; Al-Sa'aidi et al., 2015), hyperglycemia of diabetic rats (Al-Sa'aidi et al., 2014), immunity (Kalus*et al*., 2003; Hamady, 2011), as well as its antioxidant activities through enhancing the oxidant scavenger system (Salem, 2005).

The present study has been carried out to explore the possible role of thymoquinone supplementation in ameliorating the side effects of heat stress by evaluating the expression level of hsp70 gene in hepatic tissues of heat stressed male rats.

MATERIALS AND METHODS:

Experimental Animals: Mature Sprague Dewily male rats (average weight was 138±4.6g. and age was 56 days), were allowed one week to acclimatize to the animal house environment before beginning of experiment. The animals were housed under laboratory conditions (12L:12D cycles at 20-22 C°) and fed on standard laboratory food (19% protein ratio and 3000 kilocalories energy) and drinking water *ad libitum*.

Preparation of TQ Suspension: TQ (Sigma Aldrich, UK) suspension has been prepared at a dose of 50 mg/ kg bw, by dissolving 5 mg of TQ powder in 1 ml of distilled water to be used as 5 mg/1 ml/ 100 g bw (for HSTQ and TQ groups) (Kanter, 2009).

Heat Stress Protocol: During the first week, the rats have been kept at normal room temperature (21±1Cº). During the second week, male rats of heat stress groups (HS and HSTQ) were kept at room temperature which has been gradually increased (1-2 Cº for 6 hours a day) until it reaches 35±1Cº at the end of the week(Sutherland et al., 2006).

Experimental Design: The consequential alterations (each week) of liver CAT, CuZn-SOD1, GSH, and HSP70 gene expression levels have been determined in response to thymoquinone treatment of chronic heat-stressed male rats to determine the time point of thymoquinone action on liver subcellular level. Male rats were randomly divided into 4 experimental groups of 60 animals each, and treated for 6 weeks as follow: negative control male rats (C group) were daily administered with distilled water orally and reared under normal ambient temperature (21±1 ºC), non-treated heat stressed male rats

(HS group) were daily administered with distilled water orally and reared under high ambient temperature (35±1 ºC), TQ treated heat stressed male rats (HSTQ group) were daily administered with TQ suspension (50 mg/kg bw, po) and reared under high ambient temperature (35±1 ºC), and TQ treated non-stressed male rats (TQ group) were daily administered with TQ suspension (50 mg/kg bw, po) and reared under and reared under normal ambient temperature (21±1 ºC). Heat stress has been induced by exposure of male rats to high ambient temperature (at 35±1 °C for 6 hrs a day) for 6 weeks. Twenty four hours after the last administration of the treatment, for each period (1, 2, 3, 4, 5, and 6 weeks), the animals have been anaesthetized with thiopental (100 mg/ kg, i.p.), sacrificed and samples from livers were quickly dipped in DEPC solution, and frozen at -80 ºC for determination of selected gene expression levels by qRT-PCR analyses.

Total RNA Isolation: Total RNAs have been isolated from rat liver tissues according to the protocol described by the TRIzol® reagent manufacturer (Promega co. USA).. After isolation, the amount (ng/µL) and the quality of total RNA have been determined using Nanodrop UV/VIS spectrophotometer (OPTIZEN POP. MECASYS, Korea). The purity of RNA has been determined by reading the absorbance in spectrophotometer at 260 and 280 nm. Samples, whose RNA integrity number (RIN) greater than 7.0 were used in qRT-PCR experiments.

DNase Inactivation (DNase I) Treatment: The isolated RNA samples were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme) and done according to method described by Promega co., USA.

cDNA Synthesis: DNase-I treatment total RNA samples were used in cDNA synthesis step using AccuPower®RocktScript RT PreMix kit that provided from Bioneer co., Korea and done according to company instructions. The single-stranded cDNA was converted into second-strand cDNA which was used as a template for transcription reaction.

qRT-PCR Based SYBER Green I Dye Detection: qRTPCR was performed using AccuPower® Green star™ qPCR PreMix reagent kit (Bioneer, Korea) and Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea).The Syber Green based qRT-PCR PreMix reagent kit is designed for PCR amplification of cDNA for target genes (CAT, SOD1, GSTM1, and HSP70 genes) by using it's primers and (*GapdH*)

Housekeeping gene, as well as for quantification of PCR amplification copy numbers comparatively to copy numbers of Genomic DNA qRT-PCR standard curve. The Syber Green dye that was used in this kit is DNA binding dye which reacted with new copies of amplification specific segment in target and housekeeping gene. Then fluorescent signals were recorded in RT-PCR thermocycler. A genomic DNA standard curve was generated from *GapdH* gene of *Rattus norvegicus* (27.9Mbp) were taken from NCBI-Gene Bank information was approximately (~1×107) copies, and serial dilution representing (1×107, 1×106 and 1×105) gDNA copies were used as genomic DNA standard curve.

Experimental Design of qRT- PCR: For quantification of gene expression in treatment and control samples at duplicate, internal control housekeeping gene (GAPDH) was used to normalize the gene expression levels, therefore, qRT-PCR master mixes were prepared for gDNA standard curve, target genes, and GAPDH housekeeping gene. Then qPCR PreMix were added into *AccuPower* Green Star Qpcr PreMix tube. Then, real-time PCR tubes sealed by the optical adhesive film and mixed by vigorous vortexing for resuspension of PreMix pellet. The tubes centrifuge at 3,000 rpm, for 2 min, then Exicycler™ 96 Real-Time Quantitative Thermal Block instrument was started and loaded according to the kit instruction. After completion of reaction, data analysis has been performed.

Data Analysis of qRT-PCR: The housekeeping gene (*GapdH*) was represented as a normalize gene that can be used for calculation of the relative gene expression or fold change in target genes (CAT, SOD1, GSTM1, and HSP70 genes). Therefore, the quantities (Ct) of target genes were normalized with quantities (Ct) of housekeeping gene (*GapdH*) by the relative quantification gene expression levels (fold change). Livak method that described by (Livak and Schmittgen, 2001). The relative quantification method, quantities obtained from qRT-PCR experiment must be normalized in such a way that the data become biologically meaningful. In this method, one of the experimental samples is the calibrator such as (Control samples) each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate the relative expression levels. After that, the ΔCT Method with a Reference Gene was used as following equations:

ΔCT (calibrator) = CT (target, calibrator) – CT (ref, calibrator)

Ratio (target / reference) = $2^{CT\ (reference)\ -\ CT\ (target)}$

At first, the CT of the reference (ref) gene has been normalized to that of the target gene, for calibrator sample. Then the CT of the reference (ref) gene has been normalized to that of the target gene, for the test sample:

$\Delta CT\ (Test) = CT\ (target, test) - CT\ (ref, test)$

$\Delta\Delta CT = \Delta CT\ (test) - \Delta CT\ (calibrator)$

Fold change = $2^{-\Delta\Delta CT}$

Ratio (reference/target) = $2^{CT\ (reference)\ -\ CT\ (target)}$

So, the relative expression was divided by the expression value of a chosen calibrator for each expression ratio of test sample.

Statistical Analysis: Results were expressed as mean ± standard deviation. Comparisons between groups and periods values were performed using one way analysis of variance (ANOVA1) and Newman- Keuls. Differences were considered to be significant at the level of $P<0.05$. Statistical analysis was carried out using the GraphPad Prism (SAS Institute, Inc., USA).

RESULTS:

The result illustrated in figure (1) recorded significant ($P<0.05$) increase of liver CAT gene expression levels in non-treated heat stressed (HS) and TQ-treated heat stressed (TQHS) groups, starting from the first week of experiment. In HS group the elevation continued at the second week, but it gradually decreased at the third and following week to reach subnormal levels at the sixth week, whereas TQHS and TQ groups significantly ($P<0.05$) elevated than control and HS groups at the second week and continued to the last week of experiment.

Liver CuZnSOD1 gene expression levels of HS, HSTQ, and TQ groups elevated significantly ($p<0.05$) at the first three weeks. These elevations markedly increased in HS and HSTQ groups at the last three weeks, but declined in TQ group to the control level (figure 2).

Liver GSH gene expression levels of heat stressed male rats (HS and HSTQ groups) and non-stressed TQ treated male rats (TQ group) showed significant ($p<0.05$) increase in comparison with control at all experimental periods, but the elevation of TQ group was lower than heat stress groups (figure 3).

In comparison with control, liver HSP70 gene expression levels of heat stressed male rats (HS and HSTQ groups) registered significant ($p<0.05$) increase, starting at the second week of experiment and throughout the rest of experimental periods, whereas TQ group showed insignificant ($p>0.05$) difference compared with control among the experimental periods. At the last four weeks of experiment, the highest level recorded by

HSTQ group in contrast to other groups and in comparison with control at all experimental periods, but the elevation of TQ group was lower than heat stress groups (figure 4).

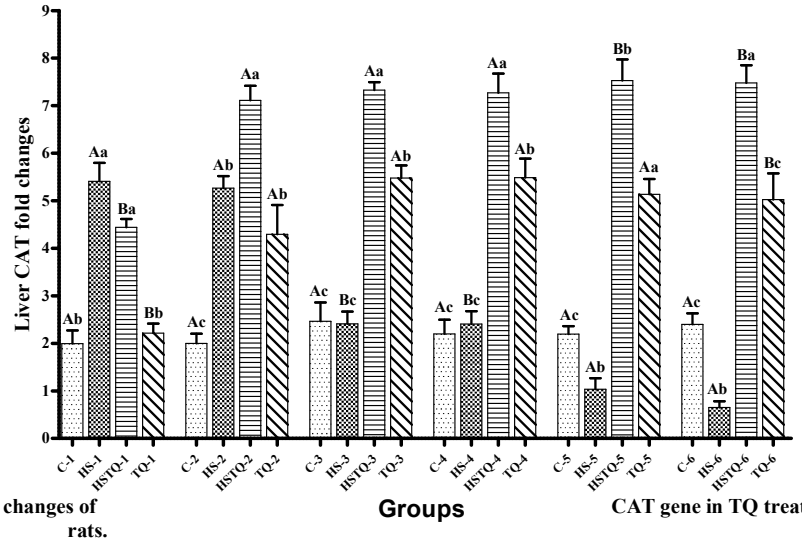

Figure (1): Liver fold changes of CAT gene in TQ treated heat stressed male rats.

C: male rats orally administered with distilled water daily for 6 weeks and reared under normal ambient temperature (21±1 ºC). HS: heat stressed male rats, orally administered with distilled water daily for 6 weeks and reared under high ambient temperature(35±1 ºC). HSTQ: heat stressed male rats, orally administered with TQ suspension (50 mg/kg, bw) daily for 6 weeks and reared under high ambient temperature(35±1 ºC). TQ: intact male rats orally administered with TQ suspension (50 mg/kg, bw) daily for 6 weeks and reared under normal ambient temperature (21±1 ºC). Data were presented as Mean ±SD of 10 observations (n=10). Different small letters denote significant difference (p<0.05) between groups for each period. Different capital letters denote significant difference (p<0.05) between periods for each group.

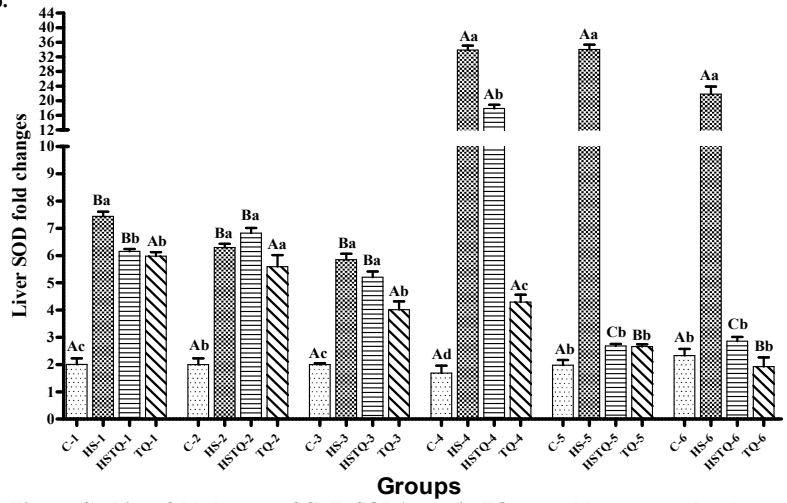

Figure (2): Liver fold changes of CuZnSOD1 gene in TQ treated heat stressed male rats.

C: male rats orally administered with distilled water daily for 6 weeks and reared under normal ambient temperature (21±1 ºC). HS: heat stressed male rats, orally administered with distilled water daily for 6 weeks and reared under high ambient temperature(35±1 ºC). HSTQ: heat stressed male rats, orally administered with TQ suspension (50 mg/kg, bw) daily for 6 weeks and reared under high ambient temperature(35±1 ºC). TQ: intact male rats orally administered with TQ suspension (50 mg/kg, bw) daily for 6 weeks and reared under normal ambient temperature (21±1 ºC). Data were presented as Mean ±SD of 10 observations (n=10). Different small letters denote significant difference (p<0.05) between groups for each period. Different capital letters denote significant difference (p<0.05) between periods for each group.

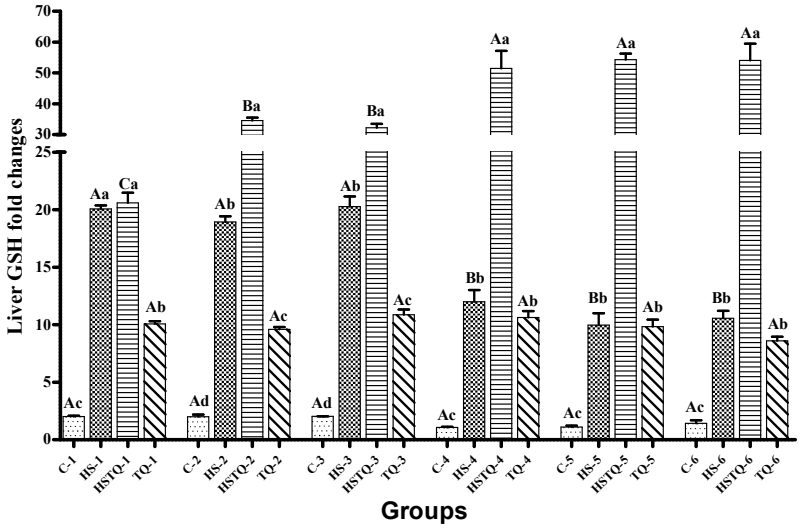

Figure (3): Liver fold changes of GSH gene in TQ treated heat stressed male rats.

C: male rats orally administered with distilled water daily for 6 weeks and reared under normal ambient temperature (21±1 ºC). HS: heat stressed male rats, orally administered with distilled water daily for 6 weeks and reared under high ambient temperature(35±1 ºC). HSTQ: heat stressed male rats, orally administered with TQ suspension (50 mg/kg, bw) daily for 6 weeks and reared under high ambient temperature (35±1 ºC). TQ: intact male rats orally administered with TQ suspension (50 mg/kg, bw) daily for 6 weeks and reared under normal ambient temperature (21±1 ºC). Data were presented as Mean ±SD of 10 observations (n=10). Different small letters denote significant difference (p<0.05) between groups for each period. Different capital letters denote significant difference (p<0.05) between periods for each group.

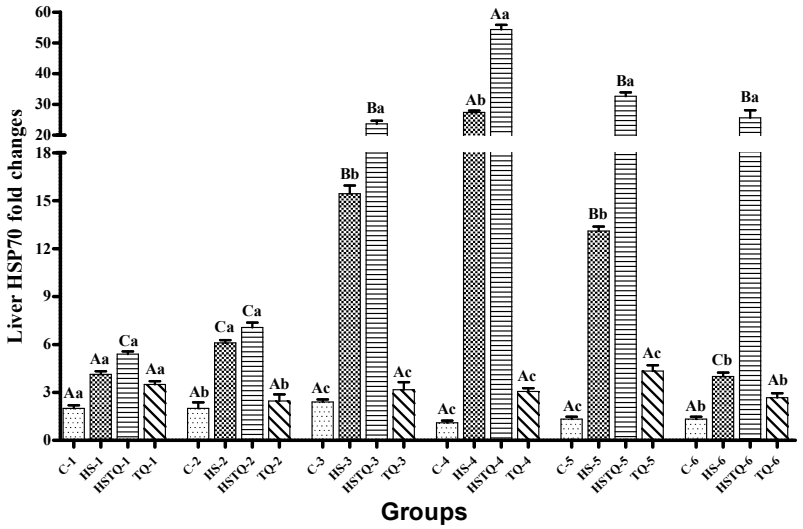

Figure (4): Liver fold changes of HSP70 gene in TQ treated heat stressed male rats.

C: male rats orally administered with distilled water daily for 6 weeks and reared under normal ambient temperature (21±1 ºC). HS: heat stressed male rats, orally administered with distilled water daily for 6 weeks and reared under high ambient temperature(35±1 ºC). HSTQ: heat stressed male rats, orally administered with TQ suspension (50 mg/kg, bw) daily for 6 weeks and reared under high ambient temperature(35±1 ºC). TQ: intact male rats orally administered with TQ suspension (50 mg/kg, bw) daily for 6 weeks and reared under normal ambient temperature (21±1 ºC). Data were presented as Mean ±SD of 10 observations (n=10). Different small letters denote significant difference (p<0.05) between groups for each period. Different capital letters denote significant difference (p<0.05) between periods for each group.

DISCUSSION

In the current study we tried to find a suggestion about the mechanism of TQ action as particular scavenger for superoxide anion produced biochemically or as general scroungers for free radicals in chronic heat stressed male rats. The outcomes of present study clarify the reported efficiency of TQ through the consolidated antioxidant properties of TQ. It is appears that TQ impact, as antioxidant, was higher in response to the presence of oxidative stress, particularly chronic heat stress, where CAT, GSH, and HSP70 increased significantly in HSTQ groups compared with non-treated non-stressed male rats (control group) and TQ-treated non-stressed male rats (TQ group). These findings were agreed with that reported by Al-Sa'aidi et al. (2016) who evaluated the potency of TQ under heat stress, but disagreed with the investigation of Mansour et al. (2002), who found that treatment of mice with the distinctive dosages of TQ (25, 50 and 100 mg/ kg/ day orally) for 5 progressive days, creates significant decrease in hepatic SOD, CAT and GSH-Px activities. Furthermore cardiovascular SOD activity was extraordinarily repressed with the higher dosages of TQ, (to be specific 50 and 100 mg/ kg), but additionally, TQ (100 mg/ kg) significantly lessened hepatic and heart lipid peroxidation as compared with control group.

Antioxidants (enzymatic and non-enzymztic) are produced inside cells to keep them safe from the impacts of reactive oxygen species (ROS), which are consistently produced in vivo both endogenously by increasing metabolic reactions and so from lipid peroxidation and exogenously from different types of stress such as heat stress (Harzallah et al., 2010). Natural antioxidants have a considerable attention, which would block or decline the side effects of these impacts. Thymoquinone, as the active compound of *Nigella sativa* crucial oil, is well known to be the the main active antioxidant of the seed (Gali-Muhtasib et al., 2008). It is obvious from these outcomes that, in heat stressed rats, the endogenous antioxidants production has been stimulated.

Al-Sa'aidi et al., (2016) have noticed that antioxidant impacts of thymoquinone could by inducing the activity of different endogenous enzymatic antioxidants for example, catalase, glutathione peroxidase and glutathione-S-transferase. From the current findings, it is obvious that treatment with TQ, under heat stress, cause the exhaustion of endogenous enzymatic and non-enzymatic antioxidants gene expressions

in hepatocytes to exceed the control level, particularly in CAT, GSH, and HSP70.

In conclusion, it can be suggested that the potency of TQ, as protective and curative effector, in hepatic oxidative damage under chronic heat stress was by the valuable role of the treatment with TQ in deceasing the of modified biochemical parameters related to the oxidative damage during heat stress, as well as to TQ efficiency in inducing the production of endogenous antioxidants. Clinical trials could be performed using thymoquinone with patients to advance its impacts information in people and to upgrade its useful impact.

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