Repeated acetaminophen administration damaged hippocampal tissue but did not affect prefrontal cortex or anxiety behaviors

Asli Karakilic[1]*, Servet Kizildag[2], Zeynep Yuce[3], Yasemin Seval Celik[4], Sevim Kandis[5],
Hemdem Rodi Bozan[6], Basar Koc[5], Guven Guvendi[7], Mehmet Ates[2], Sevinc Inan[4], Nazan Uysal[5]

[1] Department of Physiology, School of Medicine, Balıkesir University, Balıkesir, Turkey,
[2] College of Vocational School of Health Services, School of Medicine, Dokuz Eylul University, Izmir, Turkey,
[3] Department of Medical Biology and Genetics, School of Medicine, Dokuz Eylul University, Izmir, Turkey,
[4] Department of Histology and Embryology, School of Medicine, Izmir University of Economics, Izmir, Turkey,
[5] Department of Physiology, School of Medicine, Dokuz Eylul University, Izmir, Turkey,
[6] Medical student in School of Medicine, Dokuz Eylul University, Izmir, Turkey,
[7] Department of Physiology, School of Medicine, Izmir Democracy University, Izmir, Turkey,
* Email: asli.karakilic@balikesir.edu.tr

Acetaminophen is one of the most widely used over-the-counter drugs worldwide for the treatment of pain and fever. Although acetaminophen use is known to impair hippocampus-related learning and memory, its effect on anxiety is not clear. Insulin-like growth factor-1 (IGF-1) and matrix metalloproteinase-2 (MMP2) are important for cellular survival, maintenance and tissue integrity. The aim of this study was to investigate the dose-dependent effects of acetaminophen on anxiety levels as well as on hippocampus, prefrontal cortex and liver tissue. Doses of 100, 200 and 400 mg/kg acetaminophen were administered to male Sprague Dawley rats for 11 days and anxiety tests were conducted on the last day. Twenty-four hours after the last acetaminophen administration, all animals were sacrificed and hippocampus, prefrontal cortex and liver tissues were removed for analyses. Hippocampal IGF-1 and MMP2 levels were shown to decrease only at the highest dose of acetaminophen, which was accompanied by pathological changes in histology. The prefrontal cortex was not affected. Behavioral analyses also did not indicate changes in anxiety levels in the rats. Liver IGF-1 and MMP2 levels decreased in all experimental groups. Serum alanine aminotransferase and aspartate aminotransferase levels increased in the 200 mg/kg and 400 mg/kg acetaminophen groups. Our findings showed that varying doses of acetaminophen did not affect the prefrontal cortex or anxiety levels. Further research is needed to elucidate the hippocampal and hepatic protective roles of IGF-1 and MMP2 in acetaminophen toxicity and their potential use in therapeutic approaches.

Key words: acetaminophen, paracetamol, IGF-1, MMP2, hippocampus, prefrontal cortex, liver, anxiety

INTRODUCTION

Acetaminophen is one of the most commonly used over-the-counter pain relief medications administered worldwide and is considered safe to use in newborns and during pregnancy and breastfeeding. It crosses the blood-brain barrier at both therapeutic and toxic doses and is homogeneously distributed in the central nervous system (CNS) (Courad et al., 2001). However, there are many health risks associated with the use of acetaminophen, such as attention deficiency, hyperactivity syndrome, impaired motor skills and altered cognitive functions associated with spatial memory processing (Liew et al., 2014; Blecharz-Klin et al., 2017). Hepato-

toxicity and liver failure resulting from acute acetaminophen intoxication is well-documented (Fontana, 2008; Murray et al., 2008). Mechanisms associated with acetaminophen-induced liver damage include overwhelmed cytochrome P450 metabolism, mitochondrial dysfunction and glutathione depletion related to oxidative stress (Hinson et al., 2010; Letelier et al., 2011; Rofaeil et al., 2017).

Recent studies have demonstrated that acetaminophen also affects the brain, especially the hippocampus, as it can cross the blood-brain barrier (Blecharz-Klin et al., 2013; 2014; 2015; 2017); it may trigger autism and may be associated with Alzheimer's disease (Schultz, 2010, Gilmartin et al., 2015; Wu and Li, 2015). In addition, it has been shown that low dose chronic subcutaneous acetaminophen administration leads to a change in the bioamine content of the hippocampus and improves hippocampus associated learning and memory (Blecharz-Klin et al., 2014; 2017).

The prefrontal cortex is involved in the neurocircuitry underlying anxiety in humans and animals (Mathew et al., 2008; Adhikari et al., 2010) and the hippocampus is the main brain region responsible for learning and memory function. Nevertheless, regardless of its well-known function, there is accumulating evidence that it plays a role in anxiety (Engin and Treit, 2007). A functional distinction for ventral and dorsal hippocampus exists as the emotional and cognitive hippocampus, respectively. The ventral hippocampus is particularly associated with anxiety-related behaviors (Bannerman et al., 2004). Due to extensive connectivity, these functions are not exclusively attributable to hippocampal sub-regions because a sharp distinction between these sub-regions is not possible (Amaral and Witter, 1989). The dorsal and ventral hippocampal regions are comprehensively interrelated *via* the afferent and efferent projections (Witter and Amaral, 2004) and, therefore, it is difficult to discriminate their exact functions. For example, the ventral hippocampus has also clearly been shown to contribute to the spatial learning processes (de Hoz et al., 2003). Thus, to avoid confusion, we evaluated the prefrontal cortex for anxiety behavior, and the hippocampus for learning and memory function.

There are many factors affecting cell survival and cell death that are controlled by the homeostatic balance between stimulatory and inhibitory signals. For example, tyrosine phosphatase 1B triggers the anti-oxidant defense system in response to tissue damage *via* an insulin-like growth factor-1 (IGF-1)-mediated survival signal (Aguirre et al., 2000; Mobasher and Valverde 2014). IGF-1 is an anabolic hormone with endocrine, paracrine and autocrine effects, and it is mainly produced by the liver, accounting for 75% of circulating IGF-1 (Le Roith 1997; Sjogren et al., 1999). IGF-1 induces proliferation, growth and regeneration of hepatocytes, and it increases hepatocyte polarity and cell junction and extracellular matrix proteins (Perez et al., 2008; Hao et al., 2011; Lara-Diaz et al., 2017).

IGF-1 also plays crucial roles in CNS development and maturation that contribute to neuroplasticity. In response to damage, the brain has the capacity to initiate cellular repair and remodeling mechanisms. This is commonly referred to as neuroplasticity (Madathil and Saatman, 2015). IGF-1 specifically regulates hippocampal neurogenesis among other plasticity-related processes (Llorens-Martin et al., 2009). Physiological IGF-1 levels are necessary for the healthy maintenance of both liver and neuronal tissues. Additionally, IGF-1 has been reported to decrease in anxiety and depression (Llorens-Martin et al., 2010), which parallels findings from our previous study that correlated anxiety with decreased blood and prefrontal cortex IGF-1 levels (Aksu et al., 2012). Recently, we demonstrated that acetaminophen caused a decrease in blood IGF-1 levels (Ozdemir et al., 2016). However, the effect of paracetamol on brain IGF-1 levels and related behavioral parameters are still unknown.

Matrix metalloproteinases (MMPs) have many regulatory functions including the activation of growth factors, tissue regeneration, angiogenesis, remodeling of the extracellular matrix and the regulation of inflammatory processes. It is fundamental for the development and physiological maintenance of neurons (Fujioka et al., 2012; Singh et al. 2015). Optimum levels of gelatinases (MMP-2 and MMP-9) are critical for the structural and functional integrity of the basal lamina in neurons. MMP2 levels were shown to increase following acute hepatic injury and cirrhosis (Zhou et al., 2004; Bandeira et al., 2017). Gene expression of MMPs is regulated by several cytokines and growth factors such as IGF-1, nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) (Singh et al., 2015).

The aim of this study was twofold: to investigate the dose-dependent effects of acetaminophen on liver and hippocampal IGF-1 and MMP2 levels, which are both important for tissue integrity and health, and to determine its effect on behavioral parameters rendered *via* central mechanisms.

METHODS

Animals

Twenty-eight outbred adult male Sprague Dawley rats (Dokuz Eylul University School of Medicine, Experimental Animal Laboratory, Izmir, Turkey) were used in

this study. All rats were housed in individual cages with free access to water and laboratory chow. They were kept in a 12h-light/12h-dark cycle at constant room temperature (22±1°C), humidity (60%).

All experimental procedures were performed following the principles of animal care of the guidelines for the ethical use of animals in applied etiology studies and previously approved by Dokuz Eylul University School of Medicine Animal Care Committee.

Experimental design

The rats were divided into four groups: (1) Control group (n=7), (2) 100 mg/kg acetaminophen group (n=7), (3) 200 mg/kg acetaminophen group (n=7), (4) 400 mg/kg acetaminophen group (n=7). The doses of acetaminophen were determined according to our previous study (Kandis et al., 2018).

Acetaminophen was administrated as an oral solution once a day, for 11 days. An equal volume of saline was administered to the control group. On the final day, 30 min after the acetaminophen administration, anxiety levels were assessed using the open field test and elevated plus maze. Twenty-four hours following the last acetaminophen administration, all animals were sacrificed. Blood samples were collected under carbon dioxide anesthesia. Brain and liver tissues were removed; the hippocampus and prefrontal cortex were separated. Tissue samples were stored at -80°C until homogenization.

Open field test

This test apparatus, which is commonly used to assess anxiety behavior, consisted of an area of 1 × 1 m surrounded with a wall 75 cm in height, with a video camera installed 2.5 m above.

The rats were placed in the center of the open field and anxiety was measured for 5 min in a soundproof observation room illuminated with controlled light (100 lx).

Elevated plus maze

The elevated plus maze, another commonly used experimental apparatus to assess anxiety, consisted of a central platform (5 cm × 5 cm) with two open arms (50 cm long, 10 cm wide and 0.5 cm high borders) and two closed arms (50 cm long, 10 cm wide with 40 cm high walls), which were elevated 50 cm above the ground. The rats were placed on the center of the plat-

form facing the open arm and were observed for 5 min. The total number of entries into the open and closed arms as well as the time spent in the open and closed arms were measured and assessed using the Noldus Ethovision video tracking system.

Biochemical analysis

The serum corticosterone levels were analyzed with an enzyme immunoassay for corticosterone kit (catalog no: E-EL-R0269, Elabscience, Wuhan, China), with assay sensitivity 46.88 pg/mL and detection range 78.13–5000 pg/mL. IGF-1 and MMP2 levels in tissue homogenates were determined by enzyme immunoassay and calculated as mg protein per tissue (IGF-1, catalog no: EK0377, Boster, Pleasanton, CA, USA – assay sensitivity <5 pg/mL, detection range 62.5-4,000 pg/mL; MMP2, catalog no: EK0639, Boster, Pleasanton, CA, USA – assay sensitivity <10 pg/mL, detection range 156-10,000 pg/mL).

Serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) activity was measured using a colorimetric diagnostic kit (Reflotron Plus, Roche Diagnostics, Mannheim, Germany).

Histological investigation

Neuronal number was estimated in the hippocampal regions corresponding to Plates 21, 23, 25 in the rat atlas of Paxinos and Watson (1998). All sections were stained by hematoxylin and eosin (H&E).

Statistical evaluation

All statistical procedures were performed in SPSS software for Windows, version 11.0 (SPSS, Chicago, IL). Statistically significant differences between groups were analyzed using one-way-ANOVA with Bonferroni *post-hoc* test. Correlations among groups were calculated using Pearson correlation analysis. The results were presented as mean ± S.E.M., *p* values <0.05 were considered statistically significant.

RESULTS

The time spent in the middle area of the open field, walking speed and walking distance were statistically insignificant among the groups (time spent in the middle area $F_{(3,20)}=1.762$, p>0.05; walking speed $F_{(3,20)}=1.389$, p>0.05; walking distance $F_{(3,20)}=2.774$, p>0.05) (Fig. 1A).
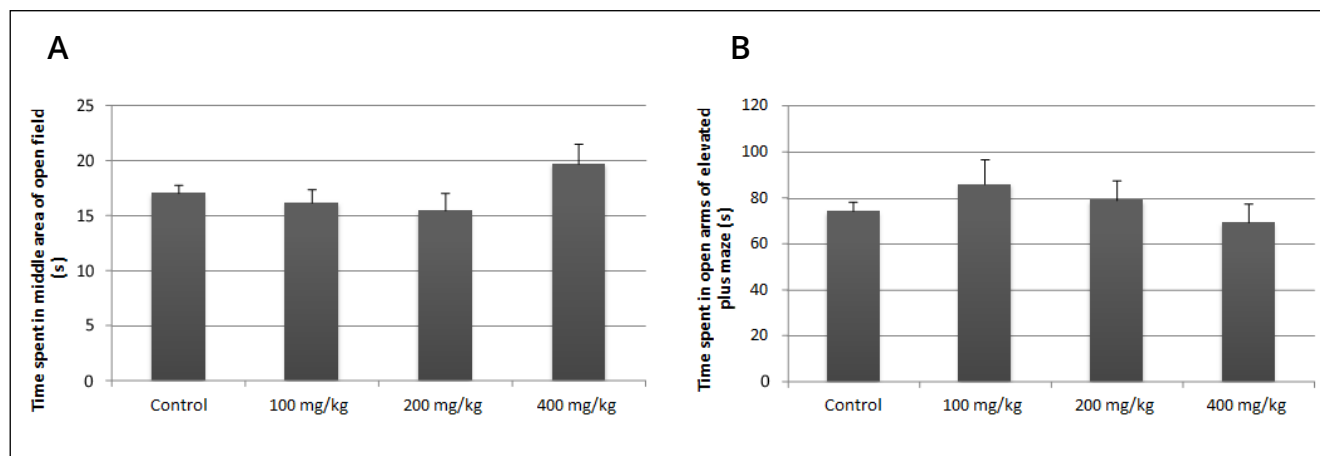
Fig. 1. Behavioral analyses. (A) Time spent in the middle area of open field (s), (B) Time spent in the open arms of elevated plus maze (s).

Additionally, no differences were observed among the groups concerning time spent in the open arms of the elevated plus maze, $F_{(3,17)}$=0.719, p>0.05 (Fig. 1B). The total number of entries into the open and closed arms of the elevated plus maze and the time spent in the closed arms were also statistically insignificant (open arm entries, $F_{(3,20)}$=0.597, p>0.05; closed arm entries, $F_{(3,20)}$=2.905, p>0.05; time spent in the closed arms, $F_{(3,20)}$=0.721, p>0.05).

IGF-1 levels in the hippocampus were found to be decreased only in the 400 mg/kg acetaminophen experimental group (compared to the control and 100 mg/kg group, p<0.05; compared to 200 mg/kg group, p<0.01), $F_{(3,16)}$=6.558, p<0.01, whereas no significant changes in prefrontal cortex IGF-1 levels were detected in any of the experimental groups when compared to the control, $F_{(3,16)}$=0.774, p>0.05 (Fig. 2A).

Liver IGF-1 levels were decreased in the 200 mg/kg and 400 mg/kg acetaminophen experimental groups (compared to control, p<0.05), $F_{(3,15)}$=6.209, p<0.01 (Fig. 2B).

MMP2 levels were found to be decreased in all acetaminophen groups for liver (100 mg/kg compared to control, p<0.05; 200 mg/kg and 400 mg/kg groups compared to control, both p<0.01), $F_{(3,15)}$=8.702, p<0.01); whereas MMP2 levels in the hippocampus were found to be decreased only in the 400 mg/kg acetaminophen group (compared to control and 100 mg/kg acetaminophen group, both p<0.05), $F_{(3,16)}$=5.508, p<0.01. No significant differences in MMP2 levels for prefrontal cortex were detected between groups, $F_{(3,16)}$=0.707, p>0.05) (Fig. 2C).

Serum ALT and AST levels were increased in the 200 mg/kg and 400 mg/kg groups when compared to the control group (p<0.001) (for ALT, $F_{(3,16)}$=24.064, p<0.001; for AST, $F_{(3,16)}$=16.178, p<0.001) (Fig. 2D).

There were no significant changes in serum corticosterone levels among any groups compared to control, $F_{(3,16)}$=0.344, p>0.05 (Fig. 2E).

Normal hippocampal morphology was observed in the control group (Fig. 3A, C). The neuronal layer of the CA region (Fig. 3E), and neurons of the dentate gyrus (Fig. 3G) displayed normal morphology. Normal histological appearance of the layers and neurons was observed in the prefrontal cortex (control group – Fig. 3I, K).

When compared to controls, histological changes in the organization of the hippocampal region were detected in the 400 mg/kg acetaminophen group (Fig. 3B, D). Disorganization of layering as well as neuronal dispersion was observed in the CA region of the hippocampus in this experimental group (Fig. 3F). Moreover, the dentate gyrus neuronal layer was thinner when compared to controls (Fig. 3H). The prefrontal cortex displayed normal histologic morphology in all experimental groups (Fig. 3J, L).

Strong positive correlations were observed between liver IGF-1 and MMP2 levels (r=0.506, p<0.05) and between prefrontal cortex IGF-1 and MMP2 levels (r=0.620, p<0.01). Hippocampal IGF-1 and MMP2 levels showed a moderate level of positive correlation (r=0.454, p<0.05).

DISCUSSION

In this study, we were able to demonstrate that only the highest dose of acetaminophen decreased IGF-1 and MMP2 levels in the hippocampus. In our experimental groups, the prefrontal cortex was not affected by acetaminophen intake. Additionally, in response to the administration of different doses of paracetamol, no
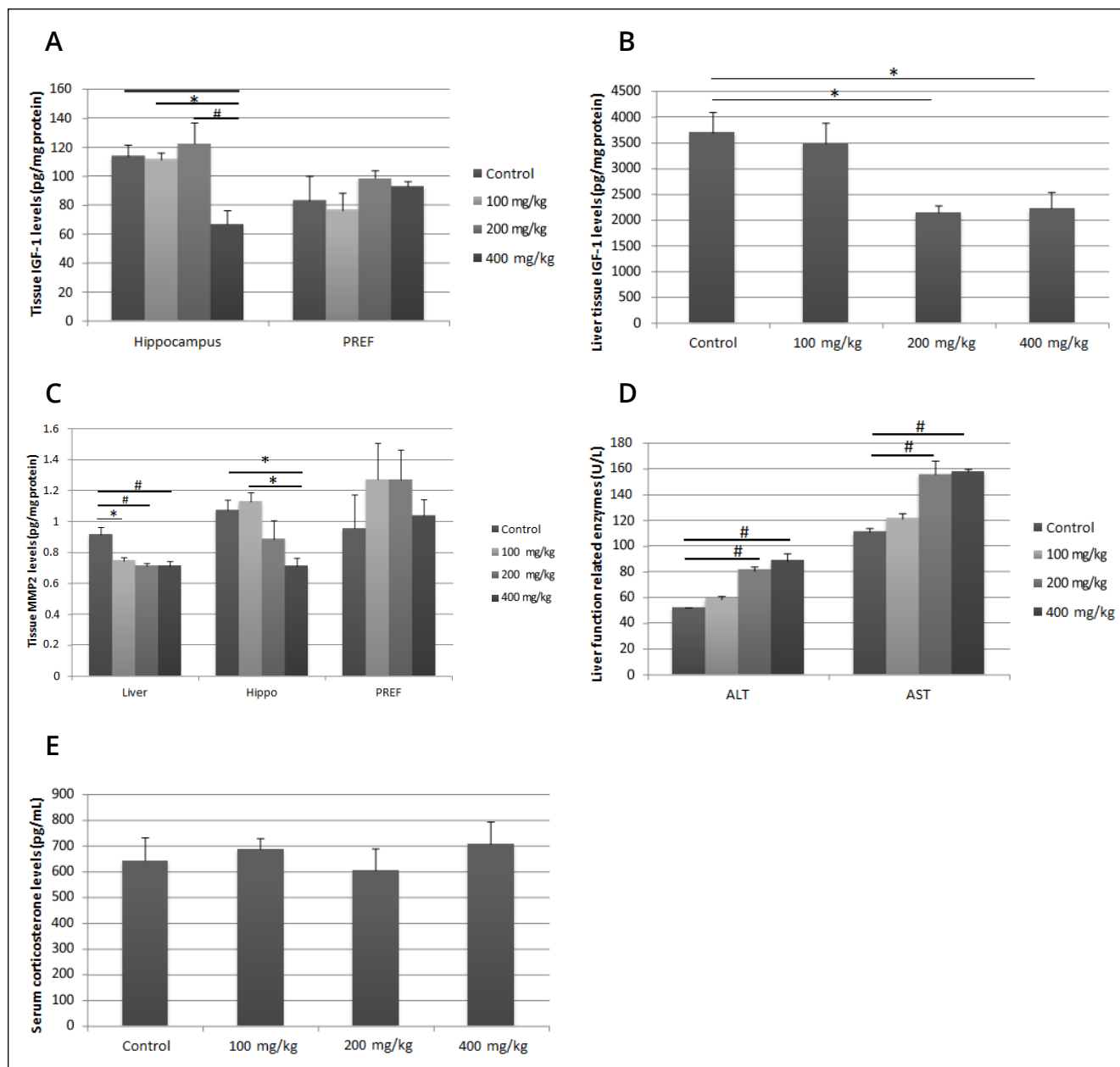
Fig. 2. Biochemical analyses. (A) Tissue IGF-1 levels, (B) Liver IGF-1 levels. (C) Tissue MMP2 levels. (D) Serum ALT and AST levels. (E) Serum corticosterone levels. IGF-1: Insulin like growth factor-1, MMP2: Matrix metalloproteinase 2, ALT: Serum alanine aminotransferase, AST: Serum aspartate aminotransferase *$p<0.05$, #$p<0.01$.

changes in anxiety levels were observed in behavioral tasks. In male Sprague Dawley rats, MMP2 levels in the liver decreased following both high and low doses of successive acetaminophen administration, whereas IGF-1 levels decreased after the successive administration of moderate and high doses (200 mg/kg and 400 mg/kg). To our knowledge, this is the first study reporting dose-dependent effects of acetaminophen on liver, prefrontal cortex and hippocampal IGF-1 and MMP2 levels.

The prefrontal cortex is one of the primary brain regions involved in emotion regulation and anxiety response. Prefrontal cortex activation results in diminished anxiety (Bishop et al., 2004). Decreased dopamine levels in the prefrontal cortex are associated with increased anxiety (Mizoguchi et al., 2010). IGF-1 has been shown to be essential for the regulation of neuronal development, maturation, proliferation and survival (Aksu et al., 2012; Ozdemir et al., 2012). We previously demonstrated that regular aerobic exer-

cise increased IGF-1 levels in both the hippocampus and prefrontal cortex of rats (Uysal et al., 2017). In addition to its well-known neuroprotective effect,
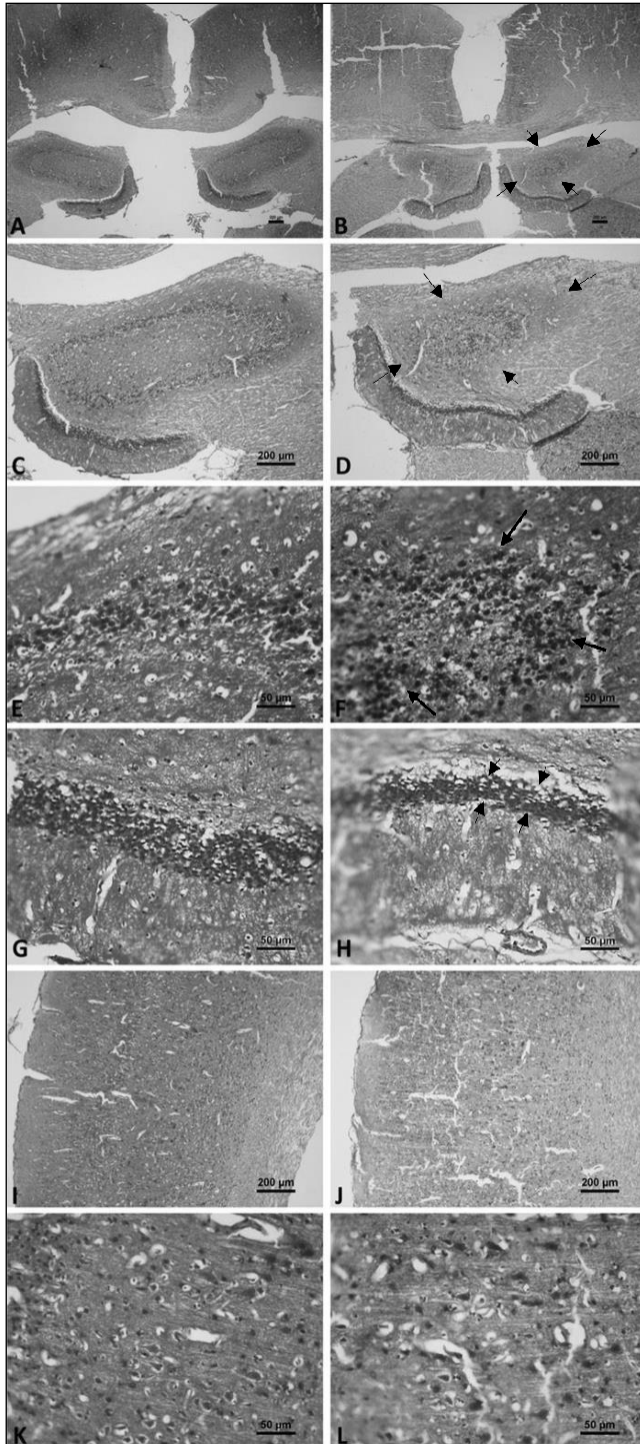

Fig. 3. Histological findings (H&E staining) in the hippocampus (A, C), CA region (E), dentate gyrus (G) and frontal cortex (I, K) of control and hippocampi (B, D), CA region (F), dentate gyrus (H) and frontal cortex (J, L) of experimental groups.

IGF-1 also has an ameliorative function on anxiety and depression (Llorens-Martin et al., 2010). For instance, increasing IGF-1 levels resulted in anxiolytic effects in both *in vitro* and *in vivo* assays (Malberg et al., 2007). Research studies have indicated that exercise-induced anxiolysis was mediated by IGF-1 (Llorens-Martin et al., 2010). On the other hand, in mice, long-term hippocampal IGF-1 deficiency was found to be related to the induction of a depressive phenotype. However, the anxiety measures remained unchanged in both the open field and elevated plus maze tasks (Mitschelen et al., 2011). Similarly, in our previous study, we demonstrated a strong positive correlation between elevated prefrontal cortex IGF-1 levels and diminished anxiety (Aksu et al., 2012). Consequently, based on this data, it can be assumed that in the CNS, the IGF-1-mediated anxiolytic effect occurs *via* the prefrontal cortex rather than the hippocampus. Therefore, based, again, on one of our previous findings that acetaminophen lowered IGF-1 levels in the blood (Ozdemir et al., 2016), we aimed to determine the effect of acetaminophen on central IGF-1 levels and anxiety behavior. In the present study, we detected no change in anxiety levels, regardless of the dose, due to acetaminophen, and also found no change in prefrontal cortex IGF-1 levels. This result is consistent with the finding that prefrontal cortex IGF-1 levels were correlated with anxiety behavior (Aksu et al., 2012). In the literature, a limited number of studies have investigated the relationship between acetaminophen use and anxiety behavior, however their results are inconsistent. Umathe et al. (2009) demonstrated a dose-dependent anxiolytic effect in mice, administering 50, 100 and 200 mg paracetamol doses 30 minutes after an i.p. drug injection. Another study, in which a low (50 mg/kg) and a high (300 mg/kg) dose of paracetamol were tested to assess effects on anxiety, concluded that a high dose of paracetamol increased anxiety levels in rats 90 minutes after the drug administration (Chen et al., 2018). In these studies, the differing time interval between acetaminophen injection and behavioral experiments might explain the discrepancy. In addition, central IGF-1 levels may not be the only mechanism explaining the effects of acetaminophen on anxiety. It is known that AM404, a metabolite of acetaminophen that is responsible for its analgesic effect, prevents the destruction of anandamide, an endocannabinoid (Dani et al., 2007). Anandamide has also been reported to yield a dose-dependent effect on anxiety (Rubino et al., 2008). As demonstrated in our study, successive acetaminophen administration had no effect on anxiety behavior and this may have been because, due to the dosage, the prefrontal cortex IGF-1 levels

had not changed. It should be noted that different receptor interactions with paracetamol metabolites in frontal or limbic brain regions might also play a role in anxiety response.

The tight regulation of tissue IGF-1 is fundamental for the control of cell cycle progression, proliferation and prevention of cell death. High or low IGF-1 levels are not compatible with physiological functioning and survival of tissues. Recent studies report that local IGF-1 protects brain tissue from oxidative damage and toxins (Pang et al., 2010, Ayadi et al., 2016). In the present study, only the high-dose acetaminophen group suffered hippocampal cell damage, which correlated with low hippocampal IGF-1 levels. However, there are conflicting reports in the literature on the effects of acetaminophen and hippocampal function. Some studies have reported that acetaminophen facilitated the emergence of autism and Alzheimer's disease (Schultz, 2010; Gilmartin et al., 2015). In contrast, Blecharz-Klin et al. (2013, 2014, 2017) reported that chronic low dose subcutaneous acetaminophen administration yielded a positive effect on the hippocampus and hippocampus-related learning. These differences may be due to specific experimental setups and differing doses. In our study, the hippocampus was affected only following high-dose acetaminophen administration and indicated that the hippocampal damage was associated with the observed decrease in IGF-1 levels. Furthermore, high-dose acetaminophen administration did not seem to affect the prefrontal cortex. Blecharz-Klin et al. (2017) investigated paracetamol use during early life and its effects on brain regions such as the hippocampus and prefrontal cortex. Our results parallel theirs, in which they reported that the hippocampus was more affected than the prefrontal cortex. On the other hand, Onaolapo and colleagues (2017) observed increased oxidative stress in the cerebral cortex following 800 mg/kg acetaminophen administration of a three-day duration. They indicated that cells were negatively affected at the microscopic level. Compared with our current study, Onaolapo et al. (2017) administered a relatively high acetaminophen dose, and this may account for the discrepancy in results.

Research studies have demonstrated that IGF-1 protected liver cells from acetaminophen-induced hepatotoxicity; furthermore, it also reversed doxorubicin-induced hepatocellular apoptosis (Alexia et al., 2004; Hwang et al., 2007). Acetaminophen increases oxidative stress in the liver, leading to oxidative damage and triggering apoptosis. In a previous report we showed that acetaminophen induced apoptosis in the liver by lowering IGF-1 levels (Ozdemir et al., 2016). In the current study, we observed that IGF-1 levels were low in regions with tissue damage. IGF-1 works as an anti-apoptotic factor by protecting cell membrane integrity (Galvan et al., 2003). The binding of IGF-1 to its receptor triggers survival signals in acetaminophen-induced hepatotoxicity (Mobasher et al., 2013).

MMPs are necessary for cell survival (Singh et al., 2015). While other MMPs localize to the extracellular area, MMP-1, MMP-2 and MMP-11 are found in the intracellular compartment and interact with other intracellular proteins (Kwan et al., 2004; Limb et al., 2005). It has been reported that MMP-2 reduced hepatic injury and increased liver regeneration (Padrissa-Altes et al., 2010). Many factors such as cytokines, growth factors and hormones control the expression of MMPs. IGF-1 is one of the hormones known to regulate MMP2 expression.

IGF-1 was shown to regulate MMP2 expression in transformed cell lines, retina and Müller glial cells (Sjogren et al., 1999; Yoon and Hurta, 2001; Zhang and Brodt, 2003; Lorenc et al., 2015). Sobrevals et al. (2010) reported that IGF-I gene transfer to cirrhotic liver induced the expression of MMP2 and other hepato-protective factors and yielded an improvement in liver function. In addition to the identification of one of the IGF-1 binding proteins as an MMP-2 substrate (Dean and Overall, 2007), Zhang et al. (2004) reported a dual regulatory role for IGF-1 in which it can up-regulate MMP-2 expression through the PI 3-kinase/Akt/mTOR signaling pathway while concomitantly transmitting a negative regulatory signal through the Raf/ERK pathway. In the present study, low IGF-1 levels were correlated with reduced tissue MMP2 levels. This may be due to the reported dual effect that IGF-1 has on MMP2 expression, which depends on several factors that can shift its regulatory role towards repression. A similar observation was reported by Bandeira et al. (2017), demonstrating that the administration of 500 mg/kg acetaminophen decreased liver MMP2 levels.

CONCLUSION

Our objective was to investigate the dose-dependent impact of acetaminophen on brain and liver IGF-1 and MMP2 levels. Only a repeated high dose of acetaminophen administration decreased IGF-1 and MMP2 levels in the hippocampus. However, prefrontal cortex IGF-1 and MMP2 levels did not change. The rats' anxiety levels were also not affected based on varying doses of acetaminophen. The behavioral results can be explained by similarly consistent IGF-1 levels in the prefrontal cortex. Our results indicate that both low and high doses of acetaminophen pathologically

affect the liver and that this is accompanied by a decrease in tissue IGF-1 and MMP2 protein expression. To our knowledge, this is the first experimental study focusing on brain levels of IGF-1 and MMP2 in rats administered acetaminophen. Reduced IGF-1 may consequently decrease MMP2 levels, adding to the increase in cellular damage. In addition to the protective roles of IGF-1 and MMP2, further research is needed to investigate other MMPs and how they relate to acetaminophen toxicity.

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