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Traumatic brain injury-induced peripheral damage: The dynamics of the inflammatory and autophagy pathway from acute to chronic stages

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Abstract

Introduction: *Traumatic brain injury (TBI) is one of the major causes of death and disability worldwide, and brings a huge burden on the quality of life of patients with TBI and the country's healthcare system. Peripheral organs, especially the kidney, and liver, may be affected by the onset of molecular responses following brain tissue damage. While secondary injury responses post TBI has been well studied in the brain, the effect/consequences of these responses in the peripheral organs have not yet been fully elucidated. Thus, our study aimed to investigate the immunoreactivity of these responses, particularly via proinflammatory cytokines and autophagy markers in the kidney and liver post-acute and chronic TBI.*

Material and methods: *Mild TBI (mTBI) and repetitive mTBI (r-mTBI) were induced in male and female 2-month-old Balb/c mice via the Marmarou weight-drop model. Liver and kidney tissues were sampled at 24 hours (acute) and 30 days (chronic) post TBI and subjected to histopathological and immunoreactivity analysis.*

Results: *Interleukin (IL)-6 levels were significantly increased in the male liver and kidney tissues in both TBI groups compared to the control group but were seen to be decreased in the female r-mTBI chronic liver and r-mTBI acute kidney. Tumor necrosis factor α (TNF-α) levels were found to increase only in the female r-mTBI chronic kidney tissue and mTBI chronic liver tissue. IL-1β levels were increased in the male and female r-mTBI liver tissues but decreased in the female mTBI kidney tissue. Inducible nitric oxide synthase (iNOS) levels were found to be significantly increased in the female mTBI acute and r-mTBI chronic kidney tissue and mTBI liver tissue, but decreased in the r-mTBI acute kidney and r-mTBI liver tissues. Beclin-1 levels were increased in male mTBI chronic and r-mTBI acute liver tissue but decreased in the r-mTBI chronic group. LC3A/B and P62/SQSTM1 levels were significantly increased in the female mTBI chronic and male r-mTBI chronic liver tissues but decreased in the male r-mTBI and female r-mTBI acute kidney tissues. Significant histopathological changes were also observed in the liver and kidney tissue which were dependent on the TBI severity, gender, and time post TBI.*

Conclusions: *The results showed that TBI may elicit peripheral molecular responses, particularly in terms of alteration in the levels of inflammatory cytokines and autophagy markers, which were gender- and time-dependent. This suggests that TBI may have a significant role in the cellular damage of the kidney and liver in both the acute and chronic phases post TBI, thus ensuring that the effects of TBI may not be confined to the brain.*

Key words: *inflammation, autophagy, peripheral tissue, neurotrauma, gender difference.*

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Introduction

Traumatic brain injury (TBI) is one of the most debilitating disorders in the world due to its high mortality and disability rate, prevalence, and significant impact on patient's quality of life. Nearly 74 million people worldwide may be suffering from various causes of TBI annually [13]. TBI causes structural injuries to neuronal tissues and physiological disruptions to various brain functions as a result of an external force on the head, which often leads to long-lasting secondary injuries [28]. Although current knowledge on the complexities of TBI pathology has progressed over the years, there is still no effective treatment against the functional outcomes that often persist post TBI, which could be caused by still lacking elucidation of the underlying secondary injury mechanism [8]. Moreover, even mild TBI (mTBI) and concussion may cause significant neuropathological changes such as multifocal axonal damage, microglia activation and microhemorrhages [9]. Among these, microglia activation has been deemed a key player in the inflammatory pathway post TBI, regardless of severity, and its activation may be temporary (acute) or permanent (chronic) [18]. However, the mechanisms underlying microglial activation and the downstream roles on the proinflammatory cytokines as well as on astrocytes may still be debatable, particularly when accounting for the threshold between repair and damage in the inflammatory pathway [1]. For example, some pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor α (TNF-α) and IL-6, which direct peripheral immune cells to the injured brain region to clear debris and promote repair, may become neurotoxic when prolonged [36]. Given that mTBI accounts for the majority of the TBI cases worldwide, understanding their inflammatory pathological changes may be more favourable and crucial for a potential therapeutic strategy.

Similarly, autophagy may also play a dual role post TBI. Autophagy may support the re-establishment of cellular homeostasis and promote the survival of injured neurons, but a previous experimental mouse study showed that autophagy may also be involved in TBI damage-inducing cell death, which may contribute to the functional outcomes in mice [35]. However, the actual impact of this autophagy response on the loss of neurons post TBI remains unclear [16]. Increasing evidence has also shown that the role of autophagy in both innate and adaptive immunity may interact with the pathogenesis of inflammation as well [26].

Interestingly, increasing evidence has indicated that TBI may not only be limited to the central nervous system (CNS), but may have far-reaching systemic/peripheral effects, whereby there may be alteration in the physiological function of peripheral organs [21,37]. There has also been evidence that these peripheral physiological and functional changes induced by TBI may even worsen the neurological outcomes post injury, in terms of possibly interacting with the inflammatory and autophagy response [7,12]. Previous studies have shown that systemic inflammation and secondary distal organ damage were observed due to a cascade of molecular and cellular events after brain injury [11]. An increase in macrophages and dying cells were also observed in another study, which showed hepatic inflammation after TBI [31].

The post-TBI inflammatory and autophagy pathway may cause bilateral damage, especially in the chronic phase, by affecting peripheral organs such as the kidney, liver, gastrointestinal system, cardiovascular system, and pulmonary system, which leaves TBI patients vulnerable to subsequent traumas and diseases [15] and therefore have a greater impact on their quality of life. Therefore, monitoring the inflammatory and autophagy process post TBI has the potential to provide crucial information about post-injury peripheral pathological pathways, which may help to predict possible worsening functional outcomes and provide a therapeutic target for them.

Thus, this study aimed to investigate the effects of the TBI-induced inflammatory and autophagy pathology in the two most crucial peripheral organs, the kidney, and liver, in both types of mild TBI, single (mTBI) and repetitive (r-mTBI), at post-acute and chronic TBI. This study also aimed to elucidate possible gender differences in the peripheral pathology post TBI.

Material and methods

Adult Balb/c mice of both male and female gender (2-month-old) were utilized in this study. Animals were housed in a controlled environment with a temperature of 24 ±2°C and humidity of 60% under a 12-h light/dark cycle. The experimental protocols for this study were performed with the approval of the Erciyes University animal experiment local ethics committee with decision number 19/127. All efforts were made to minimize animal suffering during each procedure.

Traumatic brain injury model in mice

Mild traumatic brain injury was induced in 2-month-old *Balb/c* mice (n = 7 male mice/group and n = 7 female mice/group) according to the Marmarou weight-drop model of diffuse TBI [30]. Animals were first anesthetized *via* intraperitoneal injection of 300 µl of an anaesthetic mixture containing 150 µl of 2% Rompun (Bayer, USA) and 150 µl of Ketalar (Pfizer, USA), which were prepared by dissolving them in 2 ml of 0.9% NaCl. Local

anaesthesia was also applied to the upper skin of the mice's heads before the surgical procedure. Next, the scalp of the anesthetized mouse was incised with a scalpel to expose the skull area. The skull area (along the bregma line) was cleaned, and a plastic disc was glued centrally on the bregma line. Then, the mice were placed under the Marmarou trauma device, which consisted of a pipe of 80 cm height and 30 g weight. Once the mice were positioned under the pipe appropriately (the centre of the pipe aligned with the plastic disc on the mouse's head), the 30 g weight was dropped from the top of the pipe. The force of the weight on the skull of the mice induced mild severity of TBI. As for repetitive mild traumatic brain injury (r-mTBI), a similar procedure was performed, but repeated four times across the following days; 0^{th}, 1^{st}, $3^{rd,}$ and 7^{th} day [38]. After the TBI (weight drop), the plastic helmet was removed, and the scalp was cleaned and sutured with 26 mm and 75 cm sutures (Surgisorb, UK). All mice were placed back in their respective cages after recovery. During recovery, the vital functions were monitored and the r-mTBI acute and m-TBI acute groups were sacrificed 24 h after TBI, while the r-mTBI chronic and mTBI chronic groups were sacrificed 30 days after TBI. Control (sham) groups received the scalp surgery but were not placed under the Marmarou device.

Histopathology

The liver and kidney samples were extracted from the mice and were subjected to histopathological analysis [4]. First, 5 µm thick tissue sections were cut from both the liver and kidney samples which were previously fixed in 10% formaldehyde solution and embedded in paraffin after passing through increasing series of alcohol (70%, 80%, 96%, and 100% ethanol) for dehydration of the tissue. Next, the tissue sections were stained with Harris Hematoxylin-Eosin (H&E) and visualized under light microscopy (Olympus BX53) [14].

Vacuolization, haemorrhage, glomerular degeneration, enlargement of the tubular lumen, shedding of cells in the tubule, and tubular debris were evaluated in kidney sections from female and male mice. Infiltration of inflammatory cells, sinusoidal enlargement, haemorrhage, and hepatocytes with vacuoles and/or pyknotic damage were evaluated in liver sections of female and male mice. Histopathological results in each category were scored as follows: 0 = absent, 1 = mild, 2 = moderate, and 3 = severe.

Immunohistochemistry

Immunohistochemistry (IHC) was also performed on the tissue sections using the Avidin-Biotin peroxidase method. Tissue sections were incubated with primary antibodies IL-1β (E-AB-33471, Elabscience, Houston, Texas 77079, USA), IL-6 (E-AB-40073, Elabscience, Houston, Texas 77079, USA), TNF-α (E-AB-22159, Elabscience, Houston, Texas 77079, USA), p-NF-κB (BT-MCA1291, Bioassay Technology Laboratory, China), iNOS (E-AB-70051, Elabscience, Houston, Texas 77079, USA), LC3A/B (Elabscience Biotechnology Inc, E-AB-70053), P62/SQSTM1 (Elabscience, E-AB-63539) and Beclin 1 (Shanghai YL Biotech Co. Ltd, YID0585). The immunoreactivity of these proteins was detected in the kidney and liver tissue samples according to the IHC protocols of our previous studies [2,3].

Statistical analysis

All experimental data were statistically analysed *via* GraphPad Prism (version 6.0, GraphPad Software Inc., San Diego, California, USA) and presented as mean ±SD. Immunohistochemical data were analysed with the one-way analysis of variance (ANOVA), while histopathologic data were analysed using the two-way ANOVA.

Results

Histopathological changes in the kidney and liver tissue may be dependent on TBI severity and time post TBI

Kidney

The female kidney tissues of the r-mTBI chronic group had the haemorrhage, glomerular degeneration, tubular enlargement, and tubular debris (Fig. 1, Table I; $p < 0.05$). In female mice kidneys, groups of mTBI acute, r-mTBI acute and r-mTBI chronic had only tubular enlargement ($p < 0.05$).

Renal tissue of the r-mTBI acute male group mice showed significant haemorrhage, glomerular degeneration and tubular enlargement (Fig. 1, Table II; $p < 0.05$). Chronic r-mTBI group mice had regions including vacuolization, haemorrhage and tubular pathologies when compared to other groups. In the mTBI acute group, only tubular enlargement and glomerular degeneration were more severe than the control group ($p < 0.05$) while there was no significant histopathological changes in the mTBI chronic group. For these results, there were no significant gender differences observed for histopathological analysis performed among the groups (data not presented).

Liver

The presence of haemorrhage, hollow and pycnotic hepatocyte cells were significantly increased in the

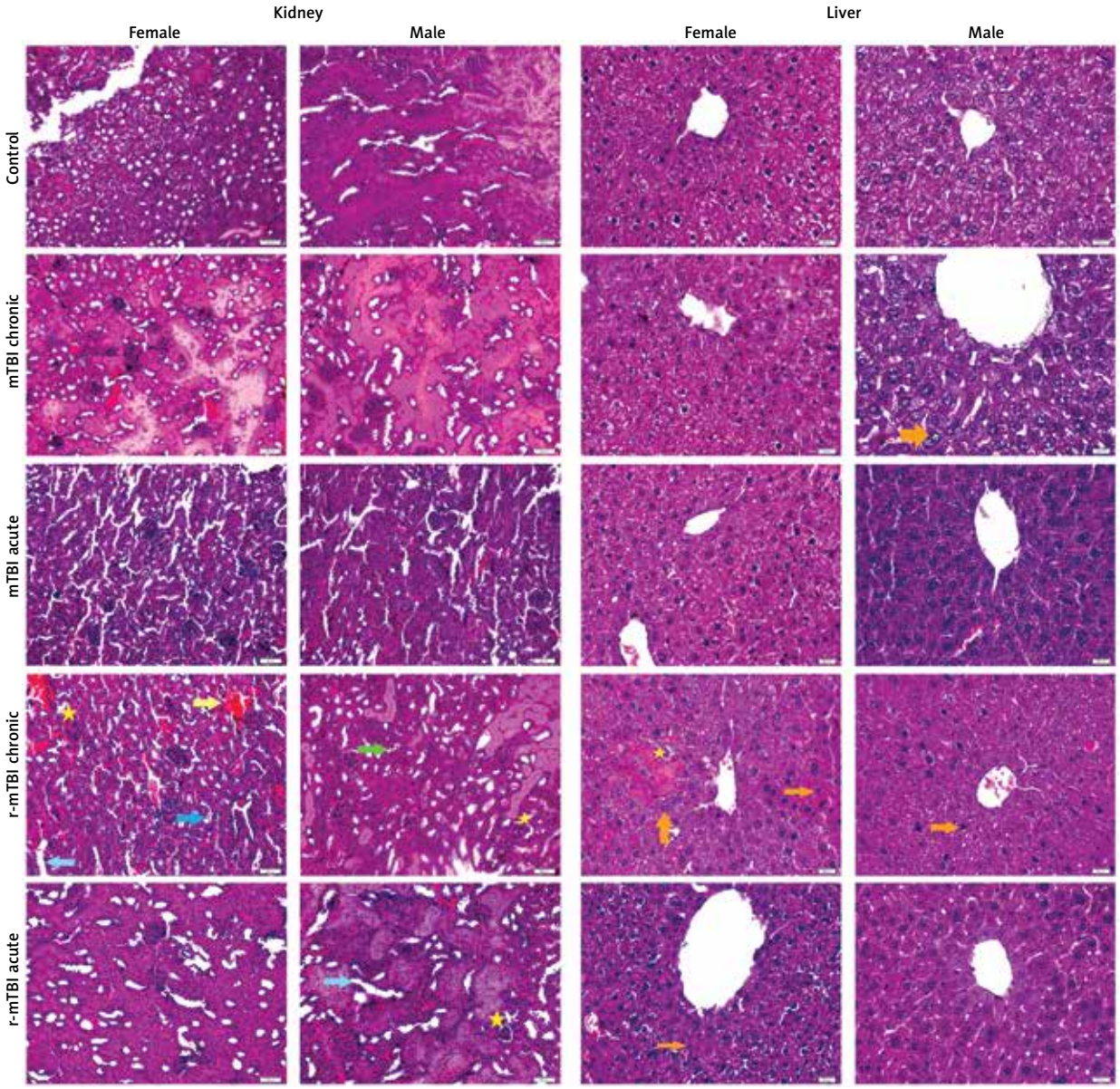


Fig. 1. Hematoxylin-eosin staining images of kidney and liver tissues. Magnification of kidney tissue 20×, bar = 50 µm (green arrow: vacuolization; yellow arrow: bleeding; star: glomerular degeneration; light blue arrow: tubular enlargement; dark blue arrow: tubular debris); magnification for liver tissue 40×, bar = 20 µm (thick orange arrow: hollow hepatocytes; thin orange arrow: pycnotic hepatocytes; yellow arrow: bleeding). mTBI – mild TBI, r-mTBI – repetitive mild TBI.

Table I. Histopathological damage score rates in female mice kidneys

Groups	Vacuolization	Haemorrhage	Glomerular degeneration	Tubular expansion	Tubular residues
Control	1.2 ±0.84	0.8 ±0.45	0.8 ±1.45	0.6 ±0.55	0.6 ±0.55
mTBI acute	1.2 ±0.84	1.2 ±0.84	1.8 ±0.45	1.2 ±0.84*	1.2 ±0.84
mTBI chronic	1.4 ±0.55	1.4 ±0.55	1.2 ±0.84	0.4 ±0.55	1.4 ±0.55
r-mTBI acute	1.2 ±0.45	1.2 ±0.84	1.8 ±0.45	1.2 ±0.84*	1.6 ±0.55
r-mTBI chronic	1.8 ±0.45	2.4 ±0.55*	2.2 ±0.84*	1.8 ±0.45*	1.8 ±0.45*

*Data are expressed as mean ±SD. Two-way ANOVA analysis of variance was applied (*p < 0.05 represents statistically significant difference).*

Table II. Histopathological damage score rates in male mice kidneys

Groups	Vacuolization	Haemorrhage	Glomerular degeneration	Tubular expansion	Tubular residues
Control	0.6 ±0.55	0.4 ±0.55	0.2 ±0.45	0.6 ±0.55	0.6 ±0.55
mTBI acute	1.2 ±0.84	1.8 ±0.45*	1.4 ±0.55*	2.2 ±0.84*	1.2 ±0.84
mTBI chronic	1.6 ±0.55	0.8 ±0.84	0.6 ±0.55	0.8 ±0.84	1.4 ±0.55
r-mTBI acute	1.4 ±0.55	2.2 ±0.84*	2.4 ±0.55*	2.4 ±0.55*	1.6 ±0.55
r-mTBI chronic	2.2 ±0.84*	1.8 ±0.45*	1.8 ±0.45*	1.4 ±0.55	1.8 ±0.45*

*Data are expressed as mean ±SD. Two-way ANOVA analysis of variance was applied (*p < 0.05 represents statistically significant difference).*

Table III. Histopathological damage score rates in female mice livers

Groups	Hollow and pycnotic hepatocytes	Haemorrhage	Sinusoidal dilation	Infiltration of inflammatory cells
Control	0.6 ±0.55	0.2 ±0.45	0.2 ±0.45	0.4 ±0.55
mTBI acute	1.2 ±0.84	0.4 ±0.55	0.4 ±0.55	0.4 ±0.55
mTBI chronic	0.8 ±0.84a	0.6 ±0.55	0.4 ±0.55	0.6 ±0.55
r-mTBI acute	2.4 ±0.54*	0.4 ±0.55	1.0 ±0.71	1.0 ±0.71
r-mTBI chronic	2.2 ±0.84*	2.2 ±0.84*[a]	0.6 ±0.55	0.4 ±0.55

*Data are expressed as mean ±SD. Two-way ANOVA analysis of variance was applied (*p < 0.05 represents statistically significant difference compared to the control group and [a]p < 0.05 represents sexual difference).*

Table IV. Histopathological damage score rates in male mice livers

Groups	Hollow and pycnotic hepatocytes	Haemorrhage	Sinusoidal dilation	Infiltration of inflammatory cells
Control	0.4 ±0.55	0.2 ±0.45	0.4 ±0.55	0.4 ±0.55
mTBI acute	1.0 ±0.71	0.4 ±0.55	0.6 ±0.55	0.4 ±0.55
mTBI chronic	2.2 ±0.84*[a]	0.8 ±0.84	1.8 ±0.84*	0.8 ±0.84
r-mTBI acute	2 ±0.71*	0.6 ±0.55	0.4 ±0.55	1.2 ±0.45
r-mTBI chronic	2.2 ±0.84*	0.4 ±0.55[a]	0.4 ±0.55	0.8 ±0.45

*Data are expressed as mean ±SD. Two-way ANOVA analysis of variance and TUKEY post hoc multiple comparison test were applied (*p < 0.05 represents statistically significant difference compared to the control group and [a]p < 0.05 represents sexual difference).*

female liver of the r-mTBI chronic group ($p < 0.05$) compared to the control group (Fig. 1, Table III).

Acute r-mTBI and chronic mTBI groups female mice showed a significant increase in the void and pycnotic hepatocytes, contrary to results in kidney tissue, the acute mTBI group mice showed no significant differences in any of the histopathological parameters in the female liver when compared to control mice.

Livers of male mice showed a significant increase in the presence of hollow and pycnotic hepatocyte cells in the mTBI chronic, r-mTBI chronic and r-mTBI acute groups (Fig. 1, Table IV). The chronic mTBI group showed a significant increase in sinusoidal dilation of the liver tissue of male mice compared to the control male group. There was a significant difference between the male and female liver, in terms of haemorrhage in the r-mTBI chronic groups and the presence of hollow and pycnotic hepatocytes in the mTBI chronic group (Tables III and IV).

TBI induces severity, gender and time-dependent inflammatory and autophagy immunoreactivity in the kidney and liver tissues

Kidney

The IL-1β expression in female kidney tissue samples was found to be decreased (by 2.34-fold, 1.71-fold, and 3.36-fold) in the mTBI chronic, mTBI acute, and r-mTBI acute groups ($p < 0.01$). On the other hand, the iNOS ($p = 0.0001$) and IL-6 ($p < 0.001$) expressions were significantly increased in the mTBI acute, r-mTBI chronic and r-mTBI acute groups (Fig. 2A, B). TNF-α expression was only significantly increased in the r-mTBI

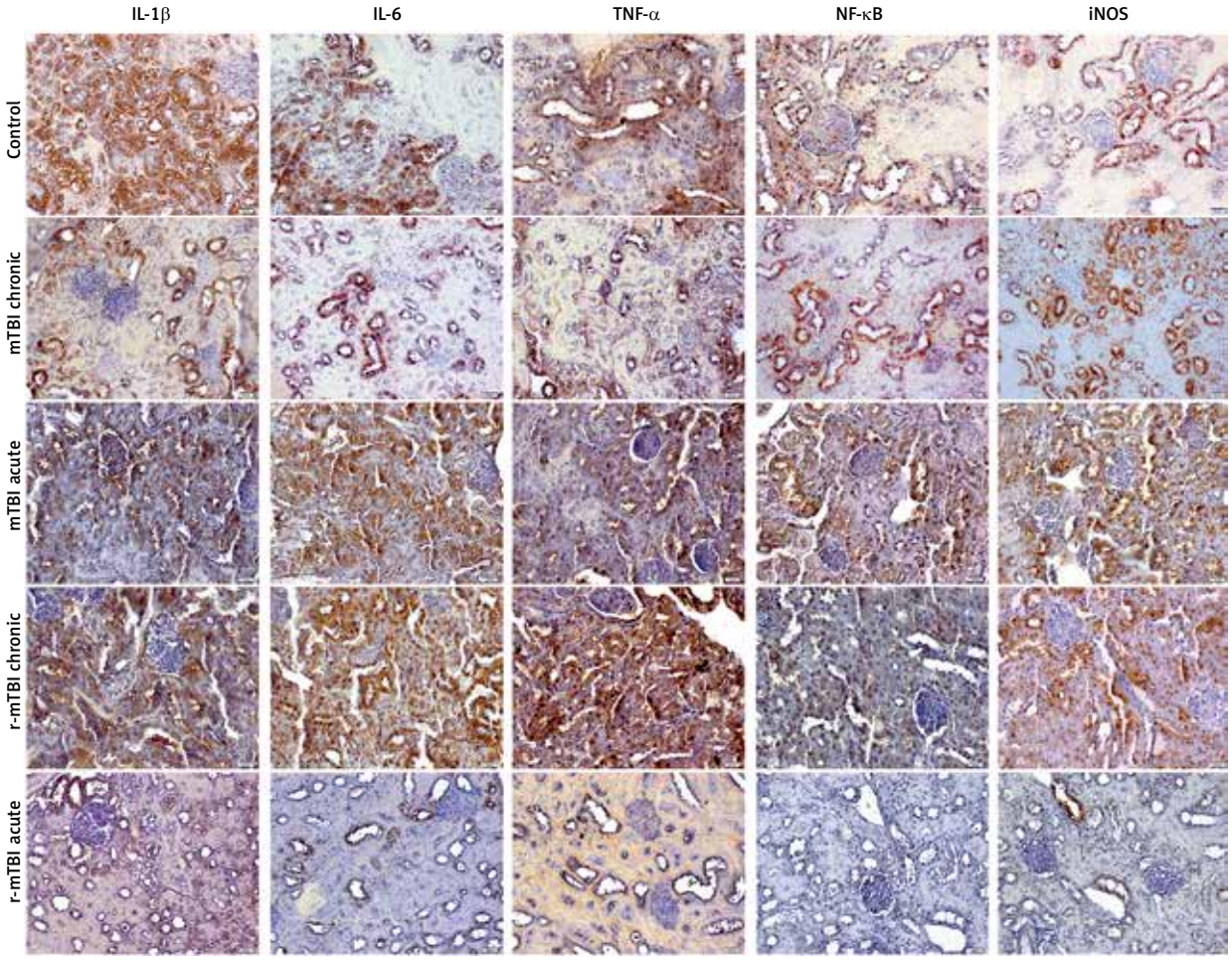


Fig. 2. Interleukin (IL)-1β, IL-6, TNF-α, NF-κB and iNOS immunostaining images in female kidney tissue samples. Magnification 40×, bar = 20 µm.

chronic group ($p < 0.01$) when compared to the control group by 1.52-fold. The NF-κB expression was found to be decreased in the r-mTBI of both chronic (2.35-fold) and acute (2.11-fold) groups compared to the control group ($p < 0.001$).

The LC3A/B expression in the female kidney tissue samples was found to be decreased in the acute r-mTBI group ($p = 0.0135$) by 1.98-fold (Fig. 3). The P62/SQSTM1 expression was found to be increased in the mTBI chronic (2.52-fold) and r-mTBI chronic (2.53-fold) groups but decreased in the r-mTBI acute group ($p < 0.0001$) by 2.06-fold. The Beclin-1 expression was observed to be significantly increased in the mTBI chronic (1.37-fold), mTBI acute (1.72-fold), and r-mTBI chronic (1.59-fold) groups ($p = 0.0025$) compared to the control group.

Both the NF-κB and iNOS expressions were seen to be decreased in the male kidney tissue samples of the mTBI acute group compared to the control group by 2.77-fold and 4.08-fold, respectively (Fig. 4). The iNOS expression was also found to be decreased in the r-mTBI acute group of the male kidney tissue by 1.86-fold compared to the control group. Like the female kidney tissue, the IL-6 expression in the male kidney tissue was also increased by 1.55-fold, 1.77-fold, 2.2-fold, and 2.12-fold in the mTBI chronic, mTBI acute, r-mTBI chronic, and r-mTBI acute groups, respectively.

The LC3A/B expression in the male kidney tissue samples was significantly increased in the mTBI chronic group compared to the control group by 2.02-fold but was found to be decreased in the r-mTBI chronic (2.85-fold) and r-mTBI acute (2.58-fold) groups ($p < 0.001$) (Fig. 5). The P62/SQSTM1 expression in the male kidney tissue was observed to be decreased in the mTBI acute, r-mTBI chronic, and r-mTBI acute groups compared to the control group ($p < 0.001$) by 2.27-fold, 1.34-fold, and 3.06-fold, respectively.

Liver

In the liver tissue, Figure 6A and B show that the IL-1β expression in the female liver tissue was signifi-

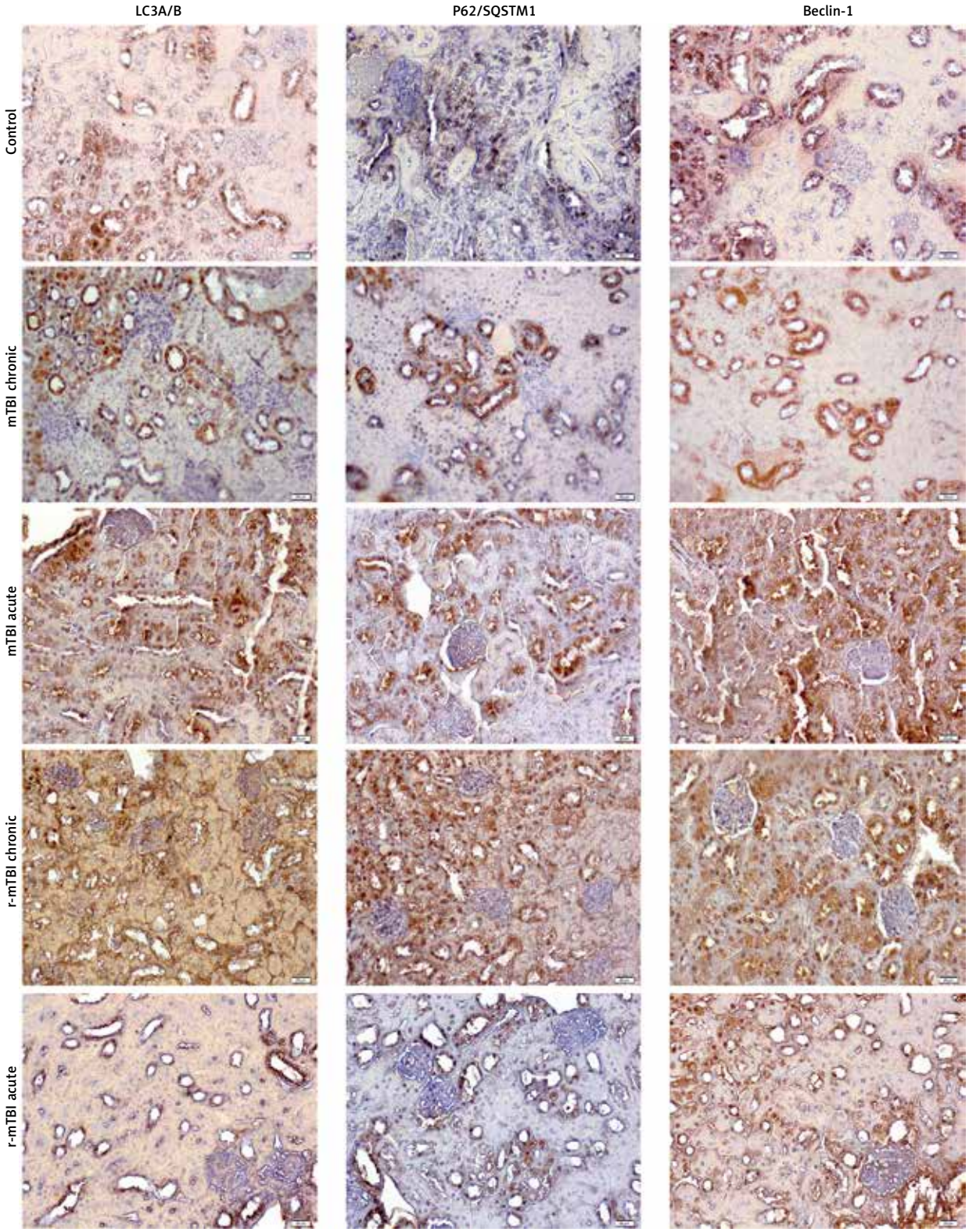


Fig. 3. LC3A/B, P62/SQSTM1 and Beclin-1 immunostaining images in female kidney tissue samples. Magnification 40×, bar = 20 µm.

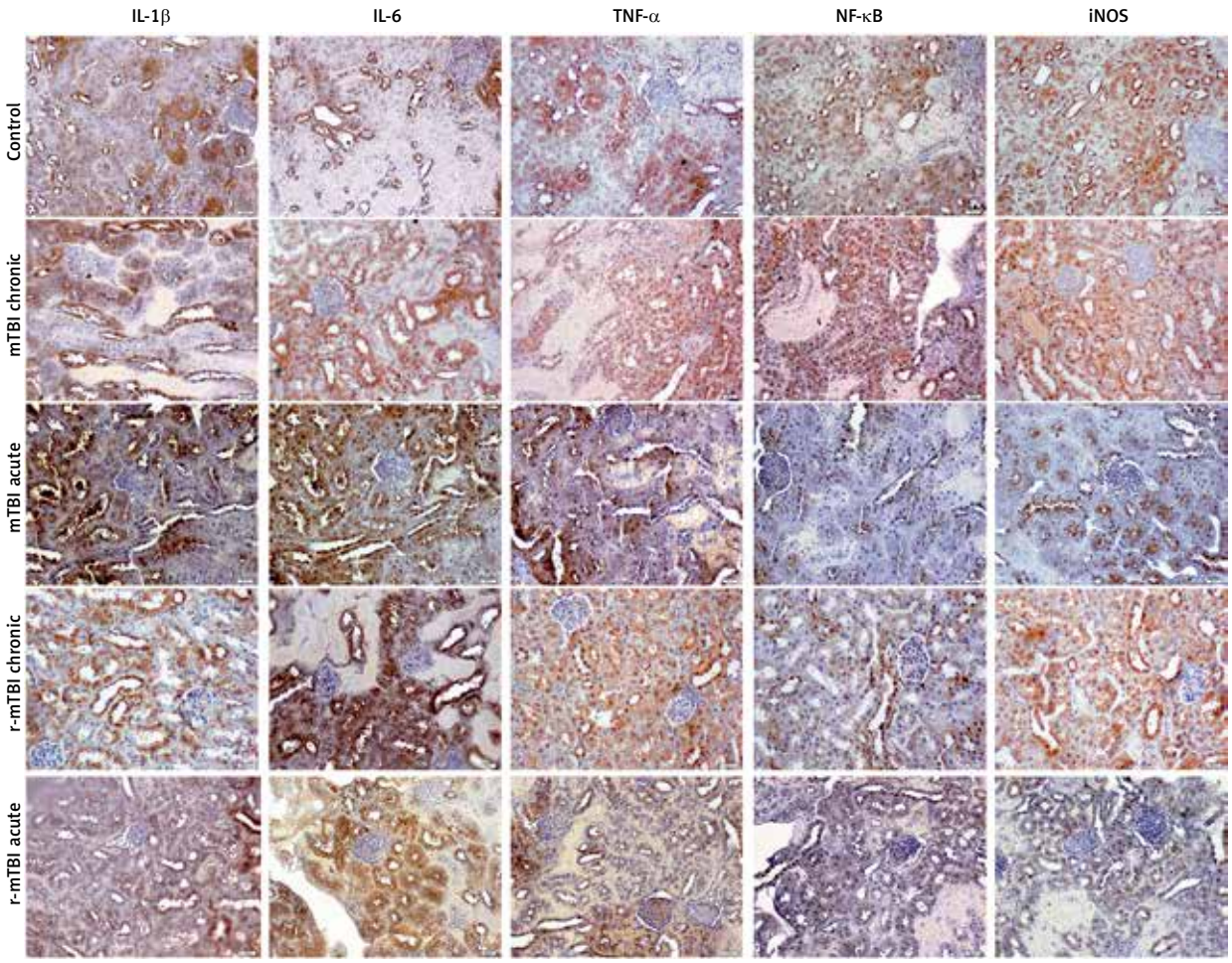


Fig. 4. Interleukin (IL)-1β, IL-6, TNF-α, NF-κB and iNOS immunostaining images in male kidney tissue samples. Magnification 40×, bar = 20 µm.

cantly increased in the mTBI chronic, r-mTBI chronic, and r-mTBI acute groups ($p < 0.0001$), by 4.19-fold, 6.23-fold and 3.24-fold, respectively. The IL-6 expression was only decreased by 2.17-fold in the r-mTBI chronic group compared to the control group ($p < 0.01$). The TNF-α expression was found to be increased in the mTBI chronic group (1.42-fold) compared to the control group but decreased in the mTBI acute group (3.1-fold) ($p < 0.001$). The NF-κB expression was seen to be increased in the mTBI chronic (2.93-fold) and mTBI acute (2.33-fold) groups compared to the control group ($p < 0.0001$). The iNOS levels were increased by 1.48-fold in the mTBI chronic and 1.55-fold in mTBI acute groups but was found to be decreased by 1.59-fold in the r-mTBI chronic and 3.36-fold in r-mTBI acute groups.

Th LC3A/B expression in female liver was noticed to be significantly increased in the mTBI chronic, r-mTBI chronic, and r-mTBI acute groups ($p < 0.0001$) by 6.13-fold, 3.54-fold and 5.48-fold, respectively (Fig. 7). The P62/SQSTM1 expression was also seen to be significantly increased in the mTBI chronic (3.37-fold) and mTBI acute (2.46-fold) groups compared to the control group ($p < 0.01$). The Beclin-1 expression was noticed to be significantly decreased in the r-mTBI chronic and r-mTBI acute groups by 1.42-fold and 2.47-fold, respectively.

In the male liver tissue, Figure 8A and B shows that the IL-6 expression was significantly increased in the mTBI chronic, mTBI acute and r-mTBI chronic groups compared to the control group, by 1.44-fold, 1.4-fold and 3.22-fold, respectively. There was a 2.31-fold, 2.8-fold and 2.04-fold increase in the IL-1β expression in the mTBI acute, r-mTBI chronic, and r-mTBI acute groups, respectively. As for TNF-α expression in the male liver tissue, a decrease of 1.5-fold in the mTBI acute, 2.25-fold in the r-mTBI chronic and 6.16-fold in the r-mTBI acute groups was observed when compared to the control group. The NF-κB expression was increased by 5.48-fold, 6.71-fold, and 7.38-fold in the mTBI acute, r-mTBI chronic and r-mTBI acute groups, respectively.

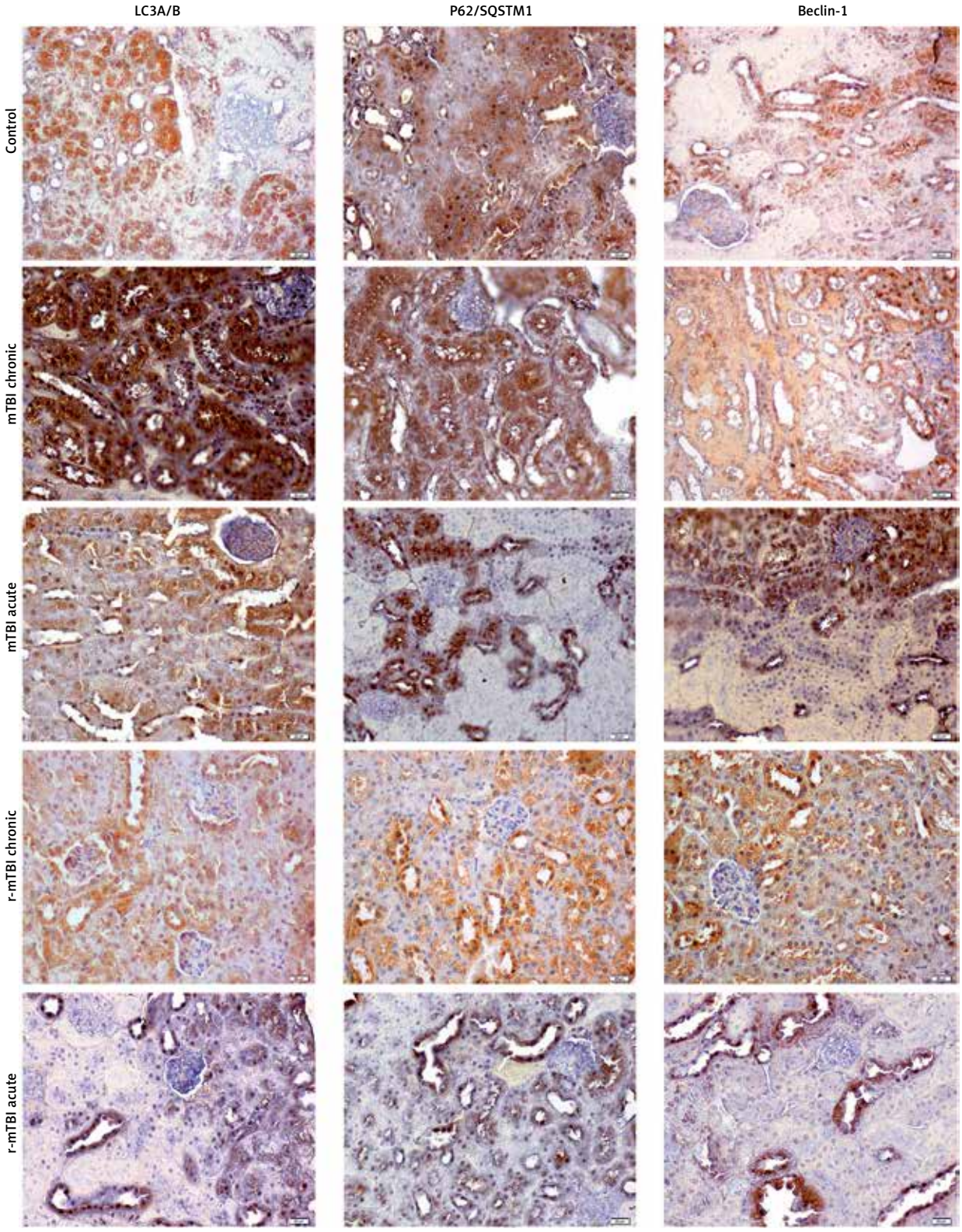


Fig. 5. LC3A/B, P62/SQSTM1 and Beclin-1 immunostaining images in male kidney tissue samples. Magnification 40×, bar = 20 µm.

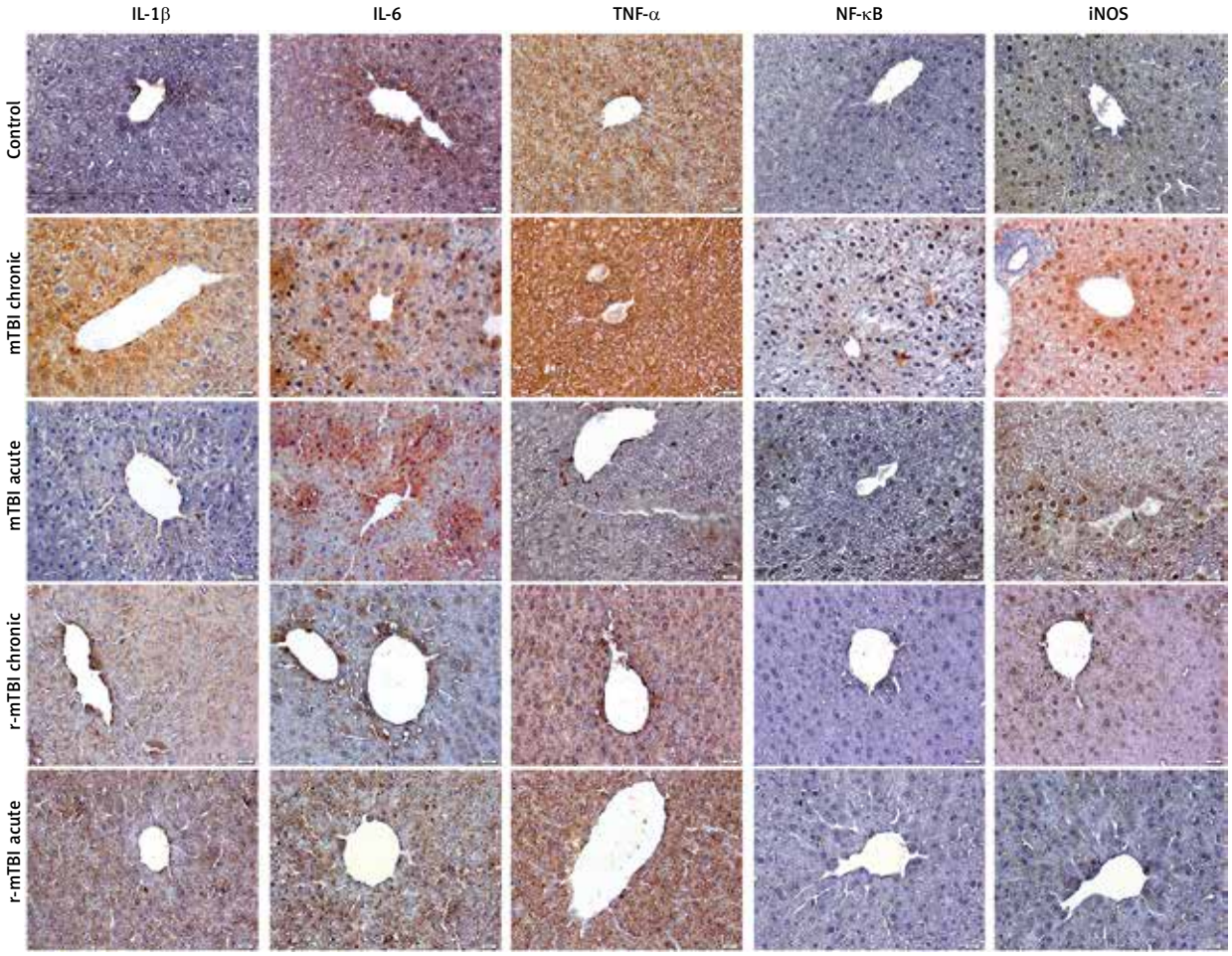


Fig. 6. Interleukin (IL)-1β, IL-6, TNF-α, NF-κB and iNOS immunostaining images in female liver tissue samples. Magnification 40×, bar = 20 µm.

In addition, an increase of 1.78-fold in the mTBI acute, 1.93-fold in the r-mTBI chronic, and 2.13-fold in r-mTBI acute groups were observed for the iNOS expression in the male liver tissue.

Figure 9 shows that the LC3A/B expression in the male liver tissue was increased in the mTBI chronic and r-mTBI chronic groups compared to the control group ($p < 0.0001$) by 6.83-fold and 8.09-fold, respectively. The P62/SQSTM1 expression was also found significantly increased in the mTBI acute (1.78-fold), r-mTBI chronic (1.67-fold), and r-mTBI acute (1.64-fold) groups ($p = 0.0168$). The Beclin-1 expression was also seen to be increased in the mTBI chronic (1.42-fold) and r-mTBI acute (2.67-fold) groups compared to the control group but were found to be decreased in the r-mTBI chronic group ($p < 0.001$) by 2.48-fold.

Discussion

The results from this study showed that mild traumatic brain injury (mTBI) causes significant changes in the histology and immunoreactivity of proinflammatory cytokines and autophagy markers in the liver and kidney tissue of female and male mice, both in the acute and chronic stages post mTBI and r-mTBI. These results suggest that the inflammatory response beginning locally in the brain tissue post TBI [17] may continue along the systemic system and activate the peripheral autophagy response, all of which may lead to damage in the peripheral organs such as the kidney and liver. This may be a possible secondary effect to the inflammatory response in the CNS, due to the brain-periphery communicative pathway [32].

The current study showed that the main pro-inflammatory cytokines; IL-1β, IL-6, TNF-α, NF-κB and iNOS, immunoreactivity in the kidney and liver were significantly influenced by TBI severity, gender, and time (acute or chronic stage) post TBI. Similar dependency was also seen in the autophagy markers and histology of the kidney and liver. TBI-related kidney and liver damage may increase the mortality rate of TBI patients [5,22]. Thus, it is crucial to monitor and predict this

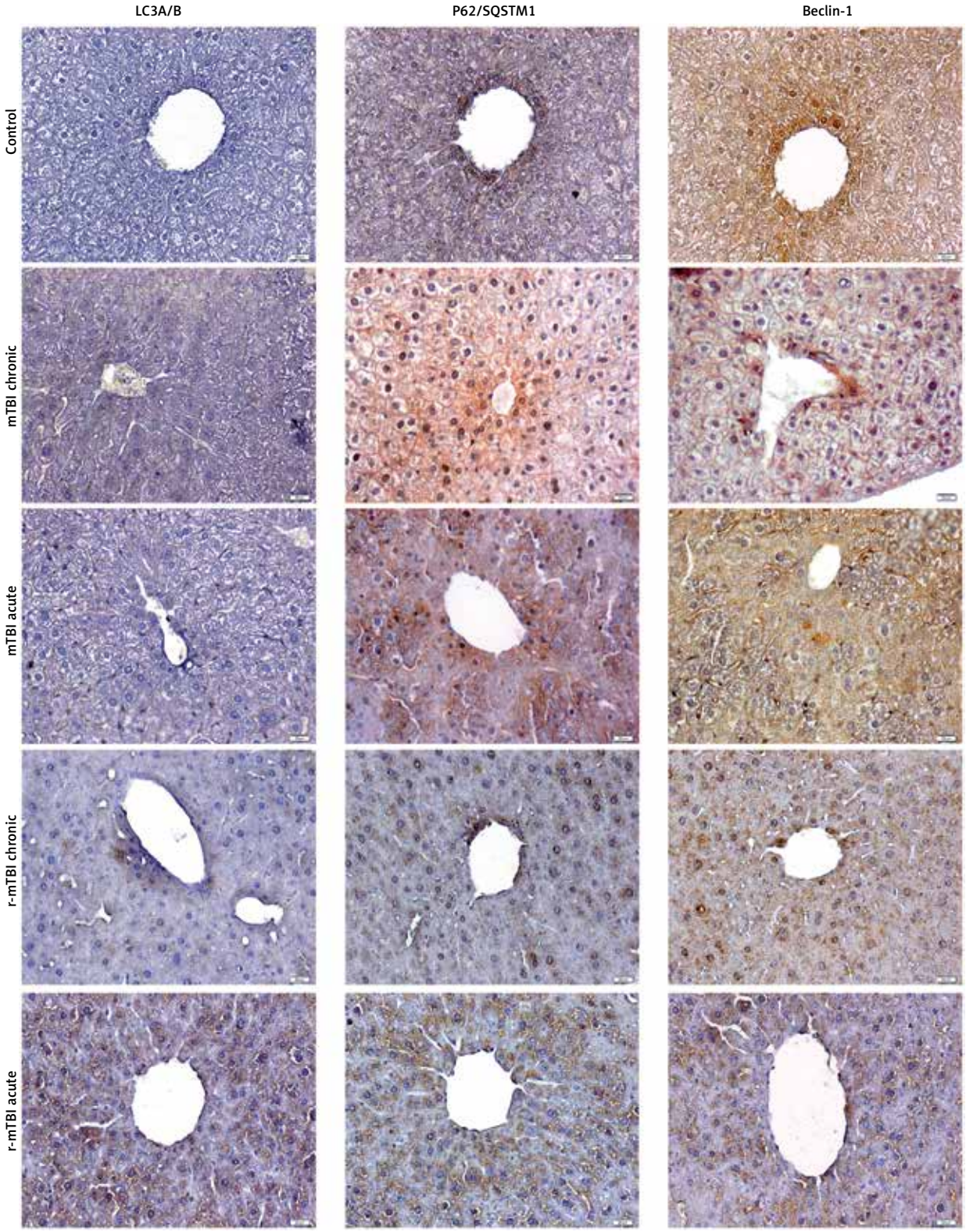


Fig. 7. LC3A/B, P62/SQSTM1 and Beclin-1 immunostaining images in female liver tissue samples. Magnification 40×, bar = 20 µm.

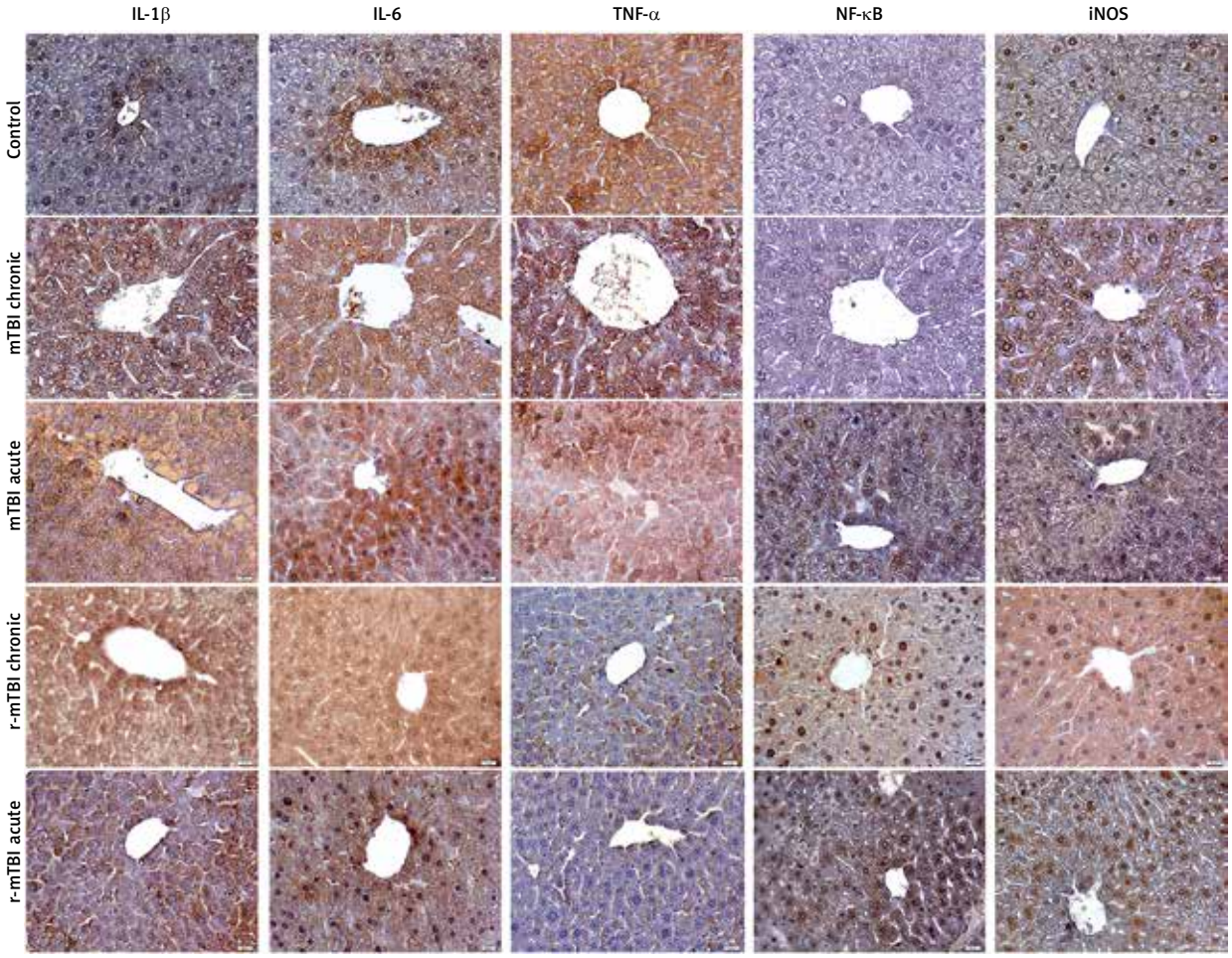


Fig. 8. Interleukin (IL)-1β, IL-6, TNF-α, NF-κB and iNOS immunostaining images in male liver tissue samples. Magnification 40×, bar = 20 µm.

damage as early as possible post TBI so that appropriate intervention could be sought after and prevent deterioration of quality of life.

In a previous study by Wang *et al.*, serum pro-calcitonin levels were shown to be an important parameter in predicting the development of acute renal failure after TBI [33]. Another study utilized the serum uric acid levels to assess the risk factor for acute renal failure development after TBI [34]. Based on the current results of this study, inflammatory and autophagy markers may also be utilized as new and more effective molecular targets to diagnose and predict the development of kidney and liver damage post TBI as they may appear as early as 24 hours post TBI, prior to any irreversible or life-threatening damage.

Interestingly, some studies have reported that the inflammatory cytokines and chemokines seen in the brain post TBI may have originated from the liver [5,39], suggesting the importance of governing and targeting the inflammatory levels of the peripheral organs in the road to TBI recovery. In the study of Campbell *et al.*, an increase in NF-kB was observed in the brain and liver following acute brain injury in a mouse model induced by intracerebral IL-1 injection. This increase in NF-κB was seen to be limited to the lesion area in the CNS but reported as occurring widely in the liver. Also, inhibition of the NF-κB activity reduced this effect on the liver [10]. This suggested that NF-κB activation caused by CNS damage may lead to inflammatory cell infiltration in the liver, which may contribute to further inflammatory activation in the brain. In support of this, this current study showed that the NF-κB immunoreactivity was significantly increased in the liver tissue of female mice in mTBI chronic and mTBI acute groups, and male mice in mTBI acute, r-mTBI acute and r-mTBI chronic groups. These effects may also translate across other inflammatory cytokines such as TNF-α, IL-6, IL-1β and iNOS. However, as this current study only looked into the peripheral organs, the dynamic influence of these inflammatory markers between the peripheral organs and the brain may still require further investigations.

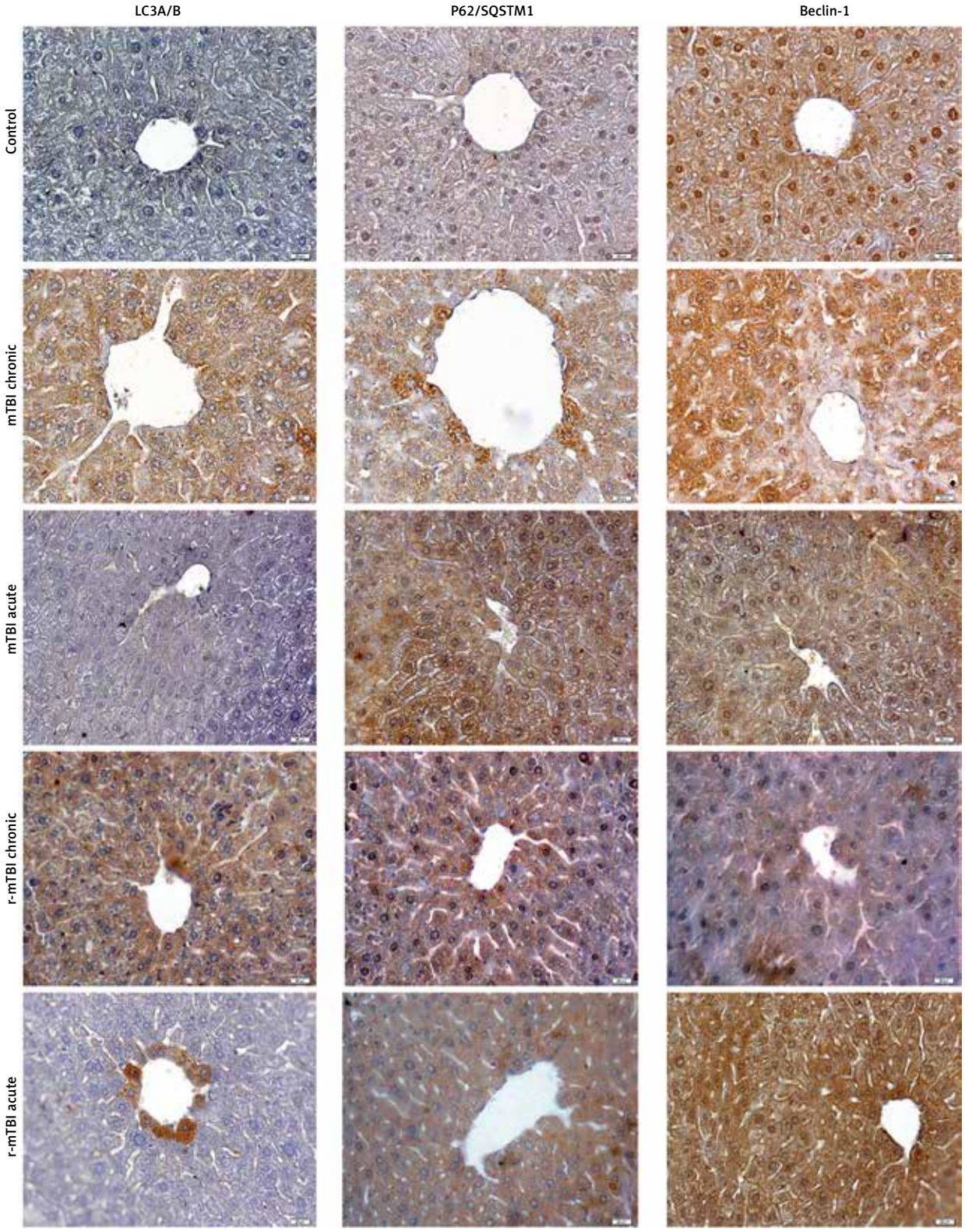


Fig. 9. LC3A/B, P62/SQSTM1 and Beclin-1 immunostaining images in male liver tissue samples. Magnification 40×, bar = 20 µm.

Autophagy may be one of the crucial causes of neuronal death contributing to cognitive disorders that develop after TBI [19]. The relationship between TBI and autophagy in peripheral organs have not yet been adequately studied. Based on the current findings, Beclin-1 immunoreactivity was significantly increased in the kidney and liver tissue of female and male mice, respectively post mTBI and r-mTBI, with variations in expression levels between the acute and chronic phases post TBI. In fact, gender influence was also observed whereby Beclin-1 was found to be significantly decreased only in the male liver of the r-mTBI chronic group, but not in the female liver. In contrast, P62/SQSTM1 immunoreactivity, another autophagy marker, was also seen to be significantly increased in the liver tissue of female mice only in the mTBI chronic and mTBI acute groups but increased in the liver tissue of male mice in the mTBI acute, r-mTBI chronic and r-mTBI acute groups. Rising P62 levels may adversely affect the recovery of the liver and brain post TBI [6], and thus may play a crucial therapeutic target.

In the study by Sarkar *et al.*, LC3 and SQSTM1 co-localization was reported to be causing neuronal death due to deterioration in the autophagy clearance [27]. This current study showed that LC3A/B immunoreactivity was significantly increased in the liver tissue of female mice in the mTBI chronic, r-mTBI chronic and r-mTBI acute groups and in the male liver tissue of mTBI chronic and r-mTBI chronic groups. This suggests that the LC3A/B and P62/SQSTM1 activities may play an interacting role in liver inflammation and damage after TBI. However, the decreasing P62/SQSTM1 immunoreactivity in the kidney tissue in both male and female TBI groups, regardless of acute or chronic stages post TBI, suggests that the autophagy pathway-related damage may be more closely related to the liver than the kidney.

In addition, histopathological deterioration was also observed in the kidney and liver tissues after TBI, dependent on the severity and time post TBI. In support of this, the study by Prus *et al.* showed that dilated vessels, perivascular lymphocytic infiltrates, dystrophic changes in the tubules and perivascular haemorrhages were observed in the kidney tissue in the early period after TBI, while perivascular and glomerular sclerosis was also detected in the chronic phases post TBI [25]. As such, this current study detected bleeding and glomerular degeneration in the male kidney tissue of the mTBI chronic group, acute tubular enlargement in the mTBI groups in both female and male mice, haemorrhage and glomerular enlargement in the kidney tissue of male mice in both acute and chronic phases in the r-mTBI groups, and residuals in the renal tubules of both female and male mice in the chronic phase after r-mTBI. These findings elucidated the severity of renal pathologies post mTBI and r-mTBI and the persistence of these pathologies into the chronic phases.

As for the liver, blood stasis in the sinusoidal capillaries of the liver tissue and adhesions to the vessel wall due to perivascular leukocytic infiltration were also observed in the acute phase after mTBI in a previous study, which showed recovery in the chronic phase [24]. In contrast, this current study observed hollow and pycnotic hepatocytes in the chronic phase after mTBI in both female and male mice, and in the acute and chronic phase of both genders post r-mTBI as well. In addition, sinusoidal dilatation was also observed in the male mice during the chronic phase after mTBI. These results may provide a new perspective on liver damage after TBI by suggesting that histopathology in the liver may persist till the chronic stages post TBI. However, further studies may be needed to evaluate the inflammatory process at the chronic stages in order to determine how the damage-recovery process of the liver may be initiated.

The results of this current study showed that gender significantly influenced the inflammatory and autophagy pathway, and to a lesser extent the histopathology, of the liver and kidney post TBI. A randomized controlled study showed that acute kidney injury may be more common in men than women post TBI [29]. However, the findings from this study show no observable significant changes in the male kidney tissue compared to female kidney tissue for histopathological changes or in most of the inflammatory and autophagy markers across the groups. In contrast, only iNOS immunoreactivity was shown to be significantly increased in the kidney tissue of the female mice, while decreased in the kidney tissue of male mice, suggesting a difference in the nitric oxide pathway. This latter finding may require further validation using other nitric pathway markers. Besides that, gender differences in the peripheral pathophysiology post TBI should also be further investigated with other markers of inflammation and possibly oxidative stress, especially since gender influences have been extensively studied and concluded in previous TBI studies [20,23].

This study provides new evidence to the literature to examine the markers of inflammation and autophagy in kidney and liver tissues and to explain the damage developed after TBI. However, one of the major limitations of this study was that the examination was limited only to the immunoreactivity of these markers in the peripheral tissue, without accounting for the changes or interaction of these immunoreactivities in the brain or cerebrospinal fluid, thus eluding the brain-periphery pathway. In future studies, stronger data should be obtained by evaluating these markers

in terms of protein levels and functions as well. Evaluation of the apoptotic activity, in addition to autophagy response, may also shed more light into the peripheral organ cellular death post TBI.

Conclusions

The results from this study showed that kidney and liver pathologies that occur after TBI may be strongly associated with the inflammation and autophagy pathway. In these tissues, IL-1β, IL-6, NF-κB, iNOS and TNF-α may play a significant role in the inflammatory process, while Beclin-1, LC3A/B and P62/SQSTM1 may be utilized to understand the autophagy-induced cellular damage in liver and kidney tissues. This study also showed that these inflammation and autophagy pathways may be influenced by the TBI severity, gender, and time since injury, which should be taken into account in future TBI therapeutic studies.

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Disclosures

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The authors report no conflict of interest.

References

1. Aguzzi A, Barres BA, Bennett ML. Microglia: scapegoat, saboteur, or something else? Science 2013; 339: 156-161.
2. Akyuz E, Doganyigit Z, Paudel YN, Kaymak E, Yilmaz S, Uner A, Shaikh MF. Increased ACh-associated immunoreactivity in autonomic centers in PTZ kindling model of epilepsy. Biomedicines 2020; 8: 113.
3. Akyuz E, Doganyigit Z, Paudel YN, Koklu B, Kaymak E, Villa C, Arulsamy A, Shaikh MF, Devinsky O. Immunoreactivity of muscarinic acetylcholine M2 and serotonin 5-HT2B receptors, norepinephrine transporter and kir channels in a model of epilepsy. Life (Basel) 2021; 11: 276.
4. Akyuz E, Polat K, Ates S, Unalmis D, Tokpinar A, Yilmaz S, Kaymak E, Doganyigit Z, Villa C. Investigating cardiac morphological alterations in a pentylenetetrazol-kindling model of epilepsy. Diagnostics (Basel) 2020; 10: 388.
5. Anthony DC, Couch Y, Losey P, Evans MC. The systemic response to brain injury and disease. Brain Behav Immun 2012; 26: 534-540.
6. Au AK, Aneja RK, Bayir H, Bell MJ, Janesko-Feldman K, Kochanek PM, Clark RSB. Autophagy biomarkers Beclin 1 and p62 are increased in cerebrospinal fluid after traumatic brain injury. Neurocrit Care 2017; 26: 348-355.
7. Beck KD, Nguyen HX, Galvan MD, Salazar DL, Woodruff TM, Anderson AJ. Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment. Brain 2010; 133: 433-447.
8. Blennow K, Brody DL, Kochanek PM, Levin H, McKee A, Ribbers GM, Yaffe K, Zetterberg H. Traumatic brain injuries. Nat Rev Dis Primers 2016; 2: 16084.
9. Blumbergs PC, Scott G, Manavis J, Wainwright H, Simpson DA, McLean AJ. Staining of amyloid precursor protein to study axonal damage in mild head injury. Lancet 1994; 344: 1055-1056.
10. Campbell SJ, Anthony DC, Oakley F, Carlsen H, Elsharkawy AM, Blomhoff R, Mann DA. Hepatic nuclear factor kappa B regulates neutrophil recruitment to the injured brain. J Neuropathol Exp Neurol 2008; 67: 223-230.
11. Civiletti F, Assenzio B, Mazzeo AT, Medica D, Giaretta F, Deambrosis I, Fanelli V, Ranieri VM, Cantaluppi V, Mascia L. Acute tubular injury is associated with severe traumatic brain injury: in vitro study on human tubular epithelial cells. J Sci Rep 2019; 9: 1-11.
12. Das M, Mohapatra S, Mohapatra SS. New perspectives on central and peripheral immune responses to acute traumatic brain injury. J Neuroinflammation 2012; 9: 236.
13. Dewan MC, Rattani A, Gupta S, Baticulon RE, Hung YC, Punchak M, Agrawal A, Adeleye AO, Shrime MG, Rubiano AM, Rosenfeld JV, Park KB. Estimating the global incidence of traumatic brain injury. J Neurosurg 2018; 130: 1080-1097.
14. Doğanyiğit Z, Okan A, Kaymak E, Pandır D, Silici S. Investigation of protective effects of apilarnil against lipopolysaccharide induced liver injury in rats via TLR 4/HMGB-1/NF-κB pathway. Biomed Pharmacother 2020; 125: 109967.
15. Faden AI, Barrett JP, Stoica BA, Henry RJ. Bidirectional brain-systemic interactions and outcomes after TBI. Trends Neurosci 2021; 44: 406-418.
16. Galluzzi L, Bravo-San Pedro JM, Blomgren K, Kroemer G. Autophagy in acute brain injury. Nat Rev Neurosci 2016; 17: 467-484.
17. Lassarén P, Lindblad C, Frostell A, Carpenter KLH, Guilfoyle MR, Hutchinson PJA, Helmy A, Thelin E. Systemic inflammation alters the neuroinflammatory response: a prospective clinical trial in traumatic brain injury. J Neuroinflammation 2021; 18: 221.
18. Loane DJ, Kumar A, Stoica BA, Cabatbat R, Faden AI. Progressive neurodegeneration after experimental brain trauma: association with chronic microglial activation. J Neuropathol Exp Neurol 2014; 73: 14-29.
19. Luo C, Tao L. The function and mechanisms of autophagy in traumatic brain injury. Adv Exp Med Biol 2020; 1207: 635-648.
20. Ma C, Wu X, Shen X, Yang Y, Chen Z, Sun X, Wang Z. Sex differences in traumatic brain injury: a multi-dimensional exploration in genes, hormones, cells, individuals, and society. Chin Neurosurg J 2019; 5: 24.
21. McDonald SJ, Sharkey JM, Sun M, Kaukas LM, Shultz SR, Turner RJ, Leonard AV, Brady RD, Corrigan F. Beyond the Brain: Peripheral Interactions after Traumatic Brain Injury. J Neurotrauma 2020; 37: 770-781.
22. Moore EM, Bellomo R, Nichol A, Harley N, Macisaac C, Cooper DJ. The incidence of acute kidney injury in patients with traumatic brain injury. Ren Fail 2010; 32: 1060-1065.
23. Munivenkatappa A, Agrawal A, Shukla DP, Kumaraswamy D, Devi BI. Traumatic brain injury: Does gender influence outcomes? Int J Crit Illn Inj Sci 2016; 6: 70.
24. Prus R, Appelhans O, Logash M, Pokotylo P, Nowicki GJ, Slusarska B. A histological and morphometric assessment of the adult and juvenile rat livers after mild traumatic brain injury. Cells 2021; 10: 1121.
25. Prus R, Pokotylo P, Logash M, Zvir T. Morphological particularities and morphometry of rats' kidneys under the effect

of experimental mild traumatic brain injury. Folia Morphol (Warsz) 2021; 80: 310-316.
26. Qian M, Fang X, Wang X. Autophagy and inflammation. Clin Transl Med 2017; 6: 24.
27. Sarkar C, Zhao Z, Aungst S, Sabirzhanov B, Faden AI, Lipinski MM. Impaired autophagy flux is associated with neuronal cell death after traumatic brain injury. Autophagy 2014; 10: 2208-2222.
28. Sato M, Chang E, Igarashi T, Noble LJ. Neuronal injury and loss after traumatic brain injury: time course and regional variability. Brain Res 2001; 917: 45-54.
29. Skrifvars MB, Moore E, Martensson J, Bailey M, French C, Presneill J, Nichol A, Little L, Duranteau J, Huet O, Haddad S, Arabi Y, McArthur C, Cooper DJ, Bellomo R, EPO-TBI Investigators and the ANZICS Clinical Trials Group. Erythropoietin in traumatic brain injury associated acute kidney injury: A randomized controlled trial. Acta Anaesthesiol Scand 2019; 63: 200-207.
30. Tweedie D, Rachmany L, Rubovitch V, Zhang Y, Becker KG, Perez E, Hoffer BJ, Pick CG, Greig NH. Changes in mouse cognition and hippocampal gene expression observed in a mild physical- and blast-traumatic brain injury. Neurobiol Dis 2013; 54: 1-11.
31. Villapol S, Kryndushkin D, Balarezo MG, Campbell AM, Saavedra JM, Shewmaker FP, Symes AJ. Hepatic expression of serum amyloid A1 is induced by traumatic brain injury and modulated by telmisartan. Am J Pathol 2015; 185: 2641-2652.
32. Villapol S. Consequences of hepatic damage after traumatic brain injury: current outlook and potential therapeutic targets. Neural Regen Res 2016; 11: 226-227.
33. Wang R, He M, Ou XF, Xie XQ, Kang Y. Serum procalcitonin level predicts acute kidney injury after traumatic brain injury. World Neurosurg 2020; 141: e112-e117.
34. Wang R, He M, Ou XF, Xie XQ, Kang Y. The predictive value of serum uric acid on acute kidney injury following traumatic brain injury. Biomed Res Int 2020; 2020: 2874369.
35. Wang YQ, Wang L, Zhang MY, Wang T, Bao HJ, Liu WL, Dai DK, Zhang L, Chang P, Dong WW, Chen XP, Tao LY. Necrostatin-1 suppresses autophagy and apoptosis in mice traumatic brain injury model. Neurochem Res 2012; 37: 1849-1858.
36. Woodcock T, Morganti-Kossmann MC. The role of markers of inflammation in traumatic brain injury. Front Neurol 2013; 4: 18.
37. Wu CL, Kor CT, Chiu PF, Tsai CC, Lian IB, Yang TH, Tarng DC, Chang CC. Long-term renal outcomes in patients with traumatic brain injury: A nationwide population-based cohort study. PLoS One 2017; 12: e0171999.
38. Xu L, Nguyen JV, Lehar M, Menon A, Rha E, Arena J, Ryu J, Marsh-Armstrong N, Marmarou CR, Koliatsos VE. Repetitive mild traumatic brain injury with impact acceleration in the mouse: Multifocal axonopathy, neuroinflammation, and neurodegeneration in the visual system. Exp Neurol 2016; 275: 436-449.
39. Yang S, Ma Y, Liu Y, Que H, Zhu C, Liu S. Elevated serum haptoglobin after traumatic brain injury is synthesized mainly in liver. J Neurosci Res 2013; 91: 230-239.