Is there any relationship between the attenuation of cerebral ischemia-reperfusion injury and serum YAP-1 levels?

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\section*{Abstract}

\textbf{Aim:} Cerebral ischemia (CI) is one of the leading causes of death worldwide. Due to the long and costly treatment process after ischemia and the need for lifelong patient care, studies in this field are important. Explaining the mechanism of damage is valuable in terms of developing preventive applications in risky persons and accelerating the treatment processes of sick patients. The aim of this study was to evaluate the relationship between serum yes-associated protein 1 (YAP-1) level, an antiapoptotic marker, and cerebral ischemia-reperfusion (CI/R) injury.

\textbf{Materials and Methods:} Wistar Albino adult (10 weeks old, 280-300 g) male rats were randomly assigned to Sham, CI, Asp+CI, and CI+Asp, with 24-hours and 7-day reperfusion periods (n=8). CI was created using the intra-arterial filament technique during 60 minutes, Asprosin (Asp) (1 \textmu g/kg/day) injections were applied 3 days before or 3 days during of 24h and 7 days reperfusion. YAP-1 levels were measured using customary ELISA kits. The Kruskal-Wallis test was used to compare differences between groups. \(p<0.05\) was considered statistically significant.

\textbf{Results:} The decrease in YAP-1 in the CI groups is statistically significant (\(p<0.05\)) compared to the sham group. The increase in YAP-1 levels in Asp groups compared with CI group is statistically significant (\(p<0.05\)).

\textbf{Conclusion:} The serum YAP-1 levels decreased due to CI/R injury. Asp, which is known to be effective in the apoptosis pathway, increased YAP-1 levels when used for protective and therapeutic purposes against CI/R injury.

\section*{Introduction}

Stroke can cause death or permanent neurological deficits due to impaired blood flow to the brain area [1]. It is the second leading cause of death among adults and leads to around five million deaths and around forty million physical disabilities worldwide annually [2]. Neurological disorders that may occur after a stroke include balance problems, hemiplegia, loss of sensation and vibration sense, numbness, decreased reflexes, visual field defects, aphasia, and apraxia [3]. Stroke can be categorised as ischemic or hemorrhagic according to the underlying pathology [4,5]. Cerebral ischemia happens when a cerebral artery is blocked, which prevents blood flow to a part of the brain. This deficiency of oxygen and energy has a harmful effect on energy-dependent events in neuronal cells [6]. Following an ischemic stroke, neurons are incapable of maintaining their homeostasis and normal transmembrane ionic gradients. Homeostasis, leading to cell death through processes such as excitotoxicity, oxidative and nitrosative stress, inflammation, and apoptosis. These pathophysiological events are interrelated and trigger each other in a positive feedback cycle that ultimately leads to neuronal destruction [7].

An ischemic brain injury can cause cell harmfull that may progress to neuron death. Neuron death is typically associated with necrosis, which exhibits well-recognised morphological features the biochemical and biophysical processes associated with necrosis have been identified. However, there is another form of cell death known as apoptosis, which plays a crucial role in the early development of tissues. Compared to cells undergoing necrosis, cells undergoing apoptosis have distinct morphological features and temporal change profiles. Apoptosis is associated with programmed cell death, where a genetic programme is activated leading to cell death, and is characterised by intermolecular DNA fragmentation. Apoptosis involves degrading DNA, cellular structure and nuclear proteins. It is also associated with the formation of apoptotic bodies and
the expression of phagocyte receptor ligand. Finally, apoptotic bodies are taken up by phagocytic cells. Apoptosis may be a major contributor to neuronal death after acute brain ischemia-reperfusion. It is not fully understood how neuronal death occurs in cerebral ischemia. Understanding the underlying mechanism is therefore clinically important for preventing and treating stroke. Molecular events that induce apoptosis in many cells, such as overproduction of free radicals, Ca\(^{2+}\) overload and excitotoxicity, are characteristic of acute brain injury. Depending on the age and location of the cells in the brain, these changes may lead to necrotic and apoptotic processes.

There are two important and distinct regions of the brain that are affected during acute cerebral ischemia and the subsequent reperfusion processes: the ischemic core and the ischemic penumbra. The ischemic core of the brain undergoes a rapid reduction in blood flow within minutes of the ischemic insult. This leads to irreversible damage and cell death. The penumbra, where damaged cells die or survive, is the therapeutic target of ischemic injury. The severity of the affected brain area varies due to differences in reduced blood flow to different areas. In addition, apoptosis may occur in the ischaemic penumbra after several hours or days, whereas necrosis begins in the ischaemic core within the first hours after the onset of acute brain injury. Given the limited treatments available for stroke, it is imperative to develop new therapeutic agents or combined treatments. Most laboratory-created therapeutic methods aim to protect neurons from pathophysiological processes, which can occur in the penumbra. So that penumbra remains the focus of therapeutic agents.

Yes-associated protein 1 (YAP-1) is a transcriptional regulator that activates genes involved in cellular survival and proliferation while repressing apoptotic genes. A key pathway regulating like apoptosis and organ growth is the Hippo signalling pathway. This signalling pathway is highly conserved in mammals [8]. YAP-1 and its paralog transcriptional co-activator with PDZ binding motif (TAZ) of transcriptional enhanced associate domains. YAP1 and TAZ work together with transcriptional enhanced associate domains to mediate the pathway that induces targeted gene expression [9]. Inactivated Hippo signaling leads to the accumulation of dephosphorylated YAP-1 in the nucleus. In the nucleus, YAP-1 upregulates transcription factors that control the expression of genes associated with anti-apoptosis, cell proliferation, re-programming, strength, epithelial-mesenchymal transition [10].

Recently, the production and secretion of asprosine (Asp), a protein structure, in white adipose tissue has been discovered. In 2016, a research team identified it as an adipokine involved in glucose release in the liver [11]. A 2017 report showed that the adipokine Asp stimulates food intake by directly activating orexigenic neurons and inhibits anorexigenic neurons via the G-protein-cAMP-PKA axis [12]. This indicates that Asp might provide novel targets for drug intervention in obesity, type 2 diabetes and metabolic disorders. In the past, it has been shown that several adipokines are involved in different physiology and pathology [13]. Previous studies have reported that pre-treatment of mesenchymal stromal cells with different adipokines can improve cell survival in the ischemia microenvironment, thus enhancing the effectiveness of mesenchymal stromal cells in the treatment of ischemia [14]. Therefore, it is possible that Asp may be able to regulate the functions and therapeutic efficacy of mesenchymal stromal cells in the cell transplantation process. Aim of this study was to investigate whether Asp treatment improves healing efficiency of cells in an ischaemic environment. In addition, we are investigating the underlying molecular mechanisms that are involved in these processes at the level of YAP-1.

In line with the information in the literature, we aimed to evaluate the changes in serum YAP-1 levels in cerebral ischemia/reperfusion injury. In addition, we aimed to evaluate the effects on serum YAP-1 levels at 24 hours and 7 days after ischemia when Asp is applied for preventive and therapeutic purposes.

Materials and Methods

Design of working groups and experimental CI modelling

A total of 64 adult male Wistar Albino rats, aged 8 weeks and weighing 280-300 g, were purchased from the Experimental Animal Research Center of Inonu University, Malatya, Turkey. The animals were handled in accordance with the Guidelines for the Care and Use of Experimental Animals. The rats were randomly assigned to cohorts and approved by the Animal Research Ethics Committee of Inonu University Faculty of Medicine (License No: 2021/19-3) on 27th September 2021. The rats were kept in a controlled environment at a temperature of 21 ±2°C with a 12/12-hour light-dark cycle. The rats have free access to water and were fed a standard laboratory chow diet. The groups sample size was determined based on a power analysis using specified values, with an α of 0.05, a power of 0.8, and a large effect size of 0.86. Although there were 8 groups, a minimum sample size of 8 participants per group was required to detect a significant difference. Therefore, the total sample size needed for analysis was determined to be 64 [15]. The study was designed to evaluate the time-dependent change of apoptosis over a period of 24 hours and 7 days. All weight-matched male rats were randomly assigned to one of eight groups (n = 8): Sham, CI, Asp+CI, and CI+Asp. The rats were individually housed for three days prior to the experiment to acclimatize to the cage stress.

Group 1- Sham Group: The animals in this group underwent sham CI surgical procedures, which involved performing all surgical procedures except for vascular occlusion. These animals were injected intraperitoneally (ip) with 1 ml of saline solution (ASP solvent) once a day, one hour after Sham CI, for 1 and 7 days according to the protocols.

Group 2- CI Group: The animals in this group were subjected to transient focal cerebral ischemia for 60 minutes. This was followed by 24 hours and 7 days of reperfusion. The rats were sacrificed 24 hours and 7 days after the start of reperfusion, as specified by the protocol.

Group 3- CI+Asp Group: The animals in this group were subjected to transient focal cerebral ischemia for 60 minutes. This was followed by 24 hours and 7 days of reperfusion. The rats were sacrificed 24 hours and 7 days after the
start of reperfusion, as specified by the protocol. One hour after reperfusion began, a single dose of 1 μg/kg/day/ml ASP was injected intraperitoneally and the animals were observed for 1 and 7 days [16]. The animals were sacrificed at the end of the appropriate period for each protocol, which was 24 hours and 7 days after reperfusion began.

**Group 4. Asp+CI Group:** animals were injected with a single dose of 1 μg/kg/day/ml ASP for three days, followed by a 60-minute temporary focal CI on the fourth day, and then reperfusion. They were sacrificed at the end of the appropriate period for each protocol, which was the start of reperfusion 24 hours and 7 days after reperfusion.

Anesthesia was induced in rats by administering 4 mg/kg of xylazine and 35 mg/kg of ketamine intraperitoneally. The body temperatures of rats were maintained at a constant range of 36.5-37 °C throughout the experiment, which was monitored by a rectal temperature probe. To continuously monitor regional cerebral blood flow, a Laser-Doppler (Moor Instruments, Axminster, Devon, UK) blood flow meter was used. The focal ischemia model was created by occluding the right middle cerebral artery using the intraluminal filament technique [17]. After 60 minutes of ischemia, the monofilament was withdrawn to initiate reperfusion at 24 hours and 7 days. The Sham CI/R group underwent the same surgical operation without filament insertion. At the end of the required period, the rats were sacrificed under anesthesia and blood samples were collected.

**Biochemical analyses**

The finaly of study 24 hours after reperfusion and 7 days after reperfusion the rats were sacrificed under anesthesia and blood tissues were collected. Serum were frozen on dry ice were stored at -80 °C under suitable conditions until the day of the analysis for ELISA analyse. Serum YAP-1 levels were measured using an ELISA kit with for rat (Cat. No: 201-11-3388; SunRed Biotechnology Company, Shanghai, China) according to the manufacturer’s instructions and results are expressed with ng/ml.

**Statistical analysis**

Statistical analysis of the data obtained from the study was performed using SPSS 22.0 software. In the data analysis, checks and correction procedures were applied to prevent missing and erroneous data and excessive/outlier value problems. Quantitative data were summarised with mean±standard deviation. When the data showed normal distribution, one-way analysis of variance was used to compare the variables between groups. Variances in intergroup comparisons Tukey’s HSD test was used because the results were homogeneous [18]. A p<0.05 was considered statistically significant.

**Results**

According to our study findings, the serum YAP-1 level of rats in the protocol with 24 hours of reperfusion after 60 minutes of cerebral ischemia is presented in Figure 1. The decrease in serum YAP-1 level in the CI/R group was statistically significant compared to the sham group (p<0.05). According to our study findings, there was no significant difference between the groups administered ASP before and after ischemia and the sham group (p<0.05).

According to our study findings, the serum YAP-1 level of rats in the protocol with a 7-day reperfusion period after 60 minutes of cerebral ischemia is presented in Figure 2. The decrease in serum YAP-1 level in the CI/R group was statistically significant compared to the sham group (p<0.05). There was no significant difference between the groups administered ASP before and after ischemia and the sham group. The increase in serum YAP-1 level was statistically significant in the ischemia group compared to the groups treated with ASP before and after ischemia (p<0.05).

Figure 1. The serum YAP-1 level of rats in the protocol with 24 hours of reperfusion after 60 minutes of cerebral ischemia. Groups marked with different letters a and b were statistically different (p<0.05). Differences between groups designated with the same letters are not statistically significant. The Kruskal-Wallis H test was used to evaluate the Western blot results of the groups. When significant differences were detected between groups, multiple pair-wise comparisons were made using the Mann-Whitney U test with Bonferroni correction.

Figure 2. The serum YAP-1 level of rats in the protocol with a 7-day reperfusion period after 60 minutes of cerebral ischemia. Groups marked with different letters a and b were statistically different (p<0.05). Differences between groups designated with the same letters are not statistically significant. The Kruskal-Wallis H test was used to evaluate the Western blot results of the groups. When significant differences were detected between groups, multiple pair-wise comparisons were made using the Mann-Whitney U test with Bonferroni correction.
During the ischemia/reperfusion process. Thus, controlling
the apoptosis pathway and monitoring its changes during
ischemia/reperfusion processes are crucial for selecting ap-
propriate treatment methods. In ischemia-reperfusion in-
jury, necrotic cells are located in the core area of ischemia,
while apoptotic cells are found in the area surrounding
the core [23]. Recent studies have shown that apoptosis,
a programmed cell death process that is a natural part
of organism development, also contributes to cell death
in various pathological conditions, including stroke [24].
Apoptosis is an energy-dependent process that results in
DNA fragmentation [25]. Most cases of ischemic brain
damage are caused by necrosis resulting from excitotoxic
damage. However, recent studies have shown that apop-
tosis also occurs in the ischemic penumbra. This area has
become a focus of interest in the process of providing neu-
ronal protection. In addition to signals from outside the
cell, there are also intracellular or mitochondria-dependent
signals that can initiate apoptosis [26]. The apoptotic pro-
cess can be initiated by intracellular signals resulting from
DNA damage, an increase in intracellular calcium levels, a
decrease in intracellular pH, and metabolic disorders [25].
In response to intracellular or extracellular signals, pro-
teases, endonucleases, and lipases are activated. Subse-
quently, chromatin condensation, cell shrinkage in the cy-
toplasm and cell body, endonucleolytic DNA fragmenta-
tion, and decreases of plasma membrane integrity occur in
the apoptotic cell. The neuronal apoptotic process crit-
ically involves the aspartyl protease family of caspasmes,
including Kaspase sisteine. Caspases activate each other,
initiating a chain of proteolytic events. While Kaspase-8,
Kaspase-9, and Kaspase-10 play an initiator role, Kaspase-
3, Kaspase-6, and Kaspase-7 play an effector role. The B-
cell lymphoma (Bel) family is a group of proteins that
regulate apoptosis, including both anti-apoptotic proteins
such as Bel-2, Bel-g, Bel-w, Bel-xl, and pro-apoptotic pro-
teins such as Bax, Bad, Bkl, and Diva. YAP-1 is an anti-
apoptotic protein that allows cancer cells to escape the
immune response through alternative pathways, thereby
suppressing apoptosis progression. YAP-1 alters protein
synthesis to modify death pathways, indicating a regula-
ory role in apoptosis beyond the known pathway [27].
Apoptosis is triggered in cells by various physiological and
pathological stimuli, leading to the activation of apoptotic
pathways. Dysregulation of apoptosis is a hallmark of
maintaining and regulating tumour growth. The studies
demonstrate that excess YAP can increase the resistance
of cancer cells to apoptosis, and that when YAP activity is
inhibited, the increase in apoptosis sensitises cancer cells
to chemotherapy [28]. It is demonstrated that YAP plays
a crucial role in controlling cancer apoptosis through mul-
tiple mechanisms and that, in the presence of apoptotic
stimuli, YAP enhances the ability of cells to reduce apop-
tosis and survive. It is noteworthy that YAP may also
function as a proapoptotic factor. When cells are under
stress due to severe DNA damage, Akt and c-Abl phos-
phorylate YAP, thereby suppressing the induction of pro-
apoptotic gene expression, and ultimately leading to the
induction of apoptosis. New therapeutic targets may be
identified by elucidating the precise mechanisms by which
YAP modulates apoptosis [29].

Our study found that the decrease in YAP-1 levels, an

Figure 3. Comparison of serum YAP-1 levels of 24 hours
and 7 days reperfusion groups after CI. Groups marked
with different letters a, b and c were statistically different
(p<0.05). Differences between groups designated with the
same letters are not statistically significant. The Kruskal-
Wallis H test was used to evaluate the Western blot results
of the groups. When significant differences were detected
between groups, multiple pair-wise comparisons were made
using the Mann-Whitney U test with Bonferroni correct-

Discussion
Stroke is currently one of the leading causes of death and
disability, with its prevalence increasing day by day [19].
Following cerebral infarction, ischaemic damage is accom-
panied by various complex pathophysiological events, such
as inflammation, excitotoxicity, oxidation, and apoptosis
[20]. The rapidly increasing inflammatory response af-

Figure 3 presents all groups in the 24-hour and 7-day pro-
tocols together. Our findings indicate that there were
no statistically significant differences between the groups
with ASP and the sham groups. The serum YAP-
1 level was statistically lower in the CI/R groups com-
pared to the sham and ASP treated groups (p<0.05). In
the groups with a CI/7-day reperfusion process, the re-
sults were statistically lower than those of the CI/24-hour
reperfusion group (p<0.05). This may be due to the induct-
ton of increased damage pathways during the reperfusion
process.

stroke in stroke is detrimental in contrast to nor-
mal physiological processes and triggers the development
of deferred brain damage [21]. Inflammation in the brain
is primarily brokered by glial cells, which produce numer-
ous cytotoxic substances. Surface receptors on microglia
[22], bind to ligands from dying cells, including heat shock
proteins and peroxiredoxins. This interaction triggers in-
tracellular signalling, which releases pro-inflammatory reg-
ulators that enhance inflammation and apoptosis, leading
to brain damage. It is argued that glial cells may worsen
brain damage following cerebral ischemia through the pro-
duction of reactive oxygen species (ROS) and the release
of anti-inflammatory cytokines [18]. ROS production be-
gins during the ischemic process and continues to increase
with the onset of reperfusion in post-ischemia treatment.
Therefore, it is essential to apply protective treatments
during reperfusion processes [17].

Apoptosis is a crucial cellular pathway affected by ROS
during the ischemia/reperfusion process. Thus, controlling
apoptosis suppressor, in CI/R groups indicates an increase in oxidative stress due to ischemia-reperfusion and increased apoptosis due to inflammation. In the 7-day reperfusion groups, increased oxidative stress decreased YAP-1 levels, which explains the time-dependent increase in apoptosis. The ASP-treated groups showed an increase in YAP-1 levels when applied before and after ischemia-reperfusion.

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Competing interests
The authors declare that they have no competing interests.

Ethical approval
This study was carried out with approval of Ethical Committee of Experimental Animals of the Faculty of Medicine in Inonu University (2021/19-3). The authors have no ethical conflicts to disclose.

Author contributions
Conceptualization, K TANBEK; methodology, K TANBEK and S SANDAL; software, K TANBEK; formal analysis, K TANBEK; data curation K TANBEK; writing, original draft preparation, K TANBEK and S SANDAL; All authors have read and agreed to the published version of the manuscript.

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