Acute Effect of Ghrelin on a Rat Model of Cerebral Ischemia/Reperfusion: Possible Role of Autophagy

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Abstract
Background: Ghrelin, a peptide 28 amino acid hormone and an endogenous ligand for growth hormone secretagogue (GHS) receptor 1a (GHS-R 1a) exerts multiple physiological functions. It has recently been investigated for its neuroprotective effects. However, the effect of ghrelin on ischemic reperfusion (I/R) injury of the brain during the acute period (1-24h) has not been the focus of much attention and the effect of ghrelin on autophagy in model of cerebral ischemia has not yet been examined. Therefore, the aim of the present study was to examine the acute effect of ghrelin in I/R rat brain model. Exploring its neuroprotective mechanisms including autophagy.

Material and Methods: 36 male albino rats, weighing 150-200g, were randomly divided into three groups: Control group: Sham operation (n=12), ischemia/reperfusion (I/R) + saline treatment vehicle group (n=12) and ischemia/reperfusion + ghrelin treatment (n=12). I/R injury of the brain was induced by occlusion of bilateral common carotid arteries for 12min. Ghrelin was administered in a dose of 0.4mg/Kg intraperitoneally after the insult. Animals were sacrificed 6 and 24hrs after ischemia (n=6 per group and per time point. Superoxide dismutase (SOD) activity and caspase-3 activity were measured. HSP70 was measured by ELISA. Gene expression of NF-κB, UCP-2, GH-R & beclin were assayed in brain tissue by Real-time RT-PCR.

Results: I/R of rat brain model caused oxidative stress resulting in significant decrease in SOD level and significant increase in NF-κB mRNA, HSP70 level, caspase-3 level and UCP-2 mRNA. With more significant increase in NF-κB, caspase-3 and UCP-2 at 24h than at 6h measured after the I/R. Also our results showed significant decrease in gene expression of beclin and ghrelin receptor in I/R of rat brain. Ghrelin treatment significantly attenuated I/R injury of rat brain as our results showed significant increase in SOD level and beclin mRNA, there were significant decrease in NF-κB mRNA, UCP-2 mRNA caspase-3 level and HSP70 level, also NF-κB mRNA, UCP-2 mRNA, caspase-3 level and beclin mRNA were returned to their control values in I/R of rat brain model with ghrelin administration. Ghrelin increased gene expression of its receptor with more significant increase at 24h than measured at 6h.

Conclusion: Our results indicate that ghrelin protected rat brain against I/R injury and its administration immediately after cerebral I/R can improve neuronal cell survival. The possible mechanisms of action including anti-apoptotic, anti-inflammatory, anti-oxidant effects of ghrelin. Also our results showed that ghrelin induced autophagy and increased gene expression of its receptor. We suggest that early ghrelin treatment may be a useful intervention after stroke in the clinic.

Key Words: Cerebral ischemia/reperfusion – Ghrelin – Autophagy.

Introduction
ISCHAEMIA-REPERFUSION injury (I/R) is a complex interplay between biochemical, cellular, and vascular endothelial factors. The clinical sequelae are organ specific, and may also involve systemic inflammatory responses [1]. Ischemia-reperfusion injury of the central nervous system (CNS) may occur after stroke, traumatic head injury, carotid endarterectomy, aneurysm repair, or deep hypothermic circulatory arrest [2]. During reperfusion, activated leukocytes interact with endothelial cells and plug capillaries, disrupt the blood-brain barrier (BBB) through the release of neutrophil-derived oxidants and proteolytic enzymes, extravasate from capillaries and infiltrate brain tissue, and release cytokines which mediate inflammation.

These processes produce an inflammatory cascade, resulting in the deterioration of the salvageable penumbra [3]. Also the mitochondria are the primary intracellular source of Reactive oxygen species (ROS), as they generate huge numbers of oxidative-reduction reactions and use massive amounts of oxygen. When anoxia is followed promptly by reperfusion, the resulting increase in oxygen supply leads to overproduction of ROS in ischemic tissues [4]. Disruption of the blood-brain barrier after I-R also results in the development of cerebral edema and increased intracranial pressure. Thus, CNS I-R injury may clinically manifest as significantly worsened sensory, motor, or cognitive functioning, or death [2,5].
The ischemic penumbra has been documented in the laboratory animal as severely hypoperfused, nonfunctional, but still viable brain tissue surrounding the irreversibly damaged ischemic core. Saving the penumbra is the main target of acute stroke therapy [6]. With ischemic stroke being the second leading cause of death and disability worldwide [7], there is a clear need for more effective therapies. Emerging evidence suggests that ghrelin may be one such potential therapy [8].

Ghrelin a 28-amino acid (aa) hormone was primary identified as endogenous ligand of the growth hormone secretagogue receptor-1a (GHSR-1a) that is principally released from X/A-like cells in the oxyntic mucosa of the stomach [9,10]. It is also produced in a wide variety of tissues, including the brain, where it might therefore act as a paracrine/autocrine factor [11]. Ghrelin is also able to cross the blood brain barrier [12]. Ghrelin exists in the plasma in a des-acylated and an acylated form, the latter of which is the result of post-translational octanoylation of pro-ghrelin by the enzyme ghrelin-O-acyltransferase (GOAT). Acylated ghrelin is the natural ligand of the growth hormone secretagogue receptor type, 1a (ghrelin receptor, GHS-R1a) through which growth hormone release is stimulated, des-acylated ghrelin does not activate GHS-R1a [13,14]. The homologous receptor, referred to as GHS-R type b (GHS-R1b), also exists but does not possess receptor activity for acylated ghrelin due to a lack of transmembrane regions 6 and 7 and thus a lack of intracellular signaling [15].

It has been demonstrated that ghrelin and the GHS receptors (GHS-1a & GHS1b), are widely expressed both in nonnervous, organs/tissues (i.e. adipose tissue, myocardium, adrenals, gonads, lung, liver, arteries, stomach, pancreas, thyroid, and kidney) [16,17] as well as in central nervous system [18,19]. It has been demonstrated that GHS-R shows different levels of expression in different tissues [16,17]. The biological actions on the heart, adipose tissue, pancreas, cancer cells and brain shared by ghrelin and the non-acylated form of ghrelin (des-octanoyl ghrelin), which does not bind GHS-R1a, represent the best evidence for the existence of a still unknown, functionally active binding site for this family of molecules [20].

Ghrelin is involved in a number of physiological functions such as regulation of food intake, body weight gain, adiposity and the control of energy homeostasis [21]. As well as, it participates in many other physiological processes such as insulin release and β-cell survival [22], circulation, cell proliferation, differentiation and apoptosis [23]. Likewise, the ghrelin has also been shown to be involved in inflammation and regulation of immune function [24] and modulation of neuronal functions [25].

Studies concerning the neuroprotective role of ghrelin were carried out in hypothalamus, in a model of rat injury, where it was able to significantly increase the number of surviving neurons and reduce the number of apoptotic neurons in CA1 area of the hippocampus [26]. Subsequently, in vitro studies on primary hypothalamic neurons exposed to oxygen-glucose deprivation protocol (OGD) further supported a neuroprotective role of ghrelin. Specifically, ghrelin exerted their actions by inhibiting generation of reactive oxygen species and stabilizing mitochondrial transmembrane potential. In addition, ghrelin-treated neurons showed an increased Bcl-2/Bax ratio, a reduced cytochrome c release, and reduced caspase-3 activation [27]. Moreover, similar to hypothalamic neurons, ghrelin exerts its neuroprotection in cortical neurons by inhibiting pro-apoptotic molecules associated with mitochondrial pathways and by activating endogenous protective molecules [28]. Ghrelin also suppresses apoptosis by increasing expression of mitochondrial uncoupling protein UCP2. UCP2 elevation effectively buffers production of reactive oxygen species, protecting the cell from oxidative stress and reducing apoptosis [29]. The effects of ghrelin on neuronal survival are not limited to neuroprotection, but also extend to cell proliferation in both embryonic and adult nervous systems [30]. Ghrelin levels have been reported to be lower in male patients after cardioembolic stroke compared with the healthy controls [31]. Given the apparent neuroprotective properties of ghrelin, restoring ghrelin levels after stroke is therefore likely to have significant beneficial outcomes.

Autophagy is a normal cellular process that is initially protective and can promote neuronal survival [32]. Generally, in the neuronal system, moderate autophagy is thought to be neuroprotective, and inadequate or defective autophagy may lead to neuronal cell death, while excess autophagy can also promote neuronal cell death [33]. There is some evidence that ghrelin may be able to encourage cell survival by stimulating autophagy in cardiomyocytes under simulated hypoxic conditions leading to a more efficient removal of damaged organelles and misfolded proteins [34]. However, this pathway has not yet been examined in vivo or in models of cerebral ischemia [35].

Another mechanism by which ghrelin may improve cell survival after ischemia is therefore by suppressing inflammation. Ghrelin treatment
in rats after an Middle cerebral artery occlusion (MCAO) reduced MCAO-induced neutrophil trafficking, TNFα, IL-6, matrix metalloproteinase 9, and nNOS, as well as apoptosis \[36\]. This treatment was associated with reduced infarct size, reduced neurological deficit, and improved 7-day survival. In this case it appears ghrelin’s mechanism of action is at least partially vagally mediated, as prior vagotomy blunted this neuroprotective effect \[36\].

The 70-kDa heat-shock proteins (HSP70s) are stress-induced molecules that are expressed in response to various types of central nervous system injuries including stroke, trauma, or neurodegenerative disorders and appear to have neuroprotective actions, but has also been linked to a deleterious role in some diseases \[37\].

The uncoupling protein, UCP-2, has been noted to possess a certain neuroprotective activity. Situated in the inner mitochondrial membrane, it is distributed in several brain regions \[38\]. Its role appears to be to dissipate the proton electrochemical gradient through the mitochondrial inner membrane. By this means, it mildly uncouples oxidative phosphorylation from respiration, decreases the inner membrane potential, and reduces ROS production, especially superoxide, by the respiratory complexes \[39\]. Thus, increased expression of this protein coincident with ischemia should reduce the production of ROS in mitochondria and confer protection on cells subject to I/R.

However, the relationship between ghrelin and I/R injury during the acute period (1-24h) has not been the focus of much attention and the effect of ghrelin on autophagy in model of cerebral ischemia has not yet been examined. Therefore, the effect of ghrelin on the brain requires further investigation. In the present study, the effect of ghrelin on autophagy and other molecular neuroprotective mechanisms are explored on the rat brain after 6h and 24h of ghrelin administration in I/R model.

**Material and Methods**

**Preparation of rat global cerebral i/r model:**

Transient forebrain I/R was induced by the modified 2-vessel occlusion model \[40\]. 36 male albino rats, weighing 150-200g, were randomly divided into three groups: Control group: Sham operation (n=12), ischemia/reperfusion (I/R) + saline treatment vehicle group (n=12) and ischemia/reperfusion + ghrelin treatment (n=12). Rats were anesthetized with 4% chloral hydrate (1mL/100g, ip) and fixed in a supine position. The bilateral common carotid arteries were exposed and were occluded using artery clamps for 12min. After ischemia for 12min, the artery clamps were removed followed by different periods of reperfusion. Rats in the control operation group were subjected to the same operation as above, except for the bilateral carotid artery occlusion. The criteria of successful ischemia included bilateral pupil dilatation and loss of righting and pain reflexes. After surgery, animals were placed into cages to recover from the anaesthesia at room temperature and were allowed food and drink. This work was done in Physiology and Biochemistry departments, Faculty of Medicine, Cairo University during Sep. 2013.

**Drug administration:**

The I/R + ghrelin group (n=12) was injected with rat ghrelin intraperitoneally (i/p) at a dose of 0.4mg/kg \[26\] at the onset of ischemia. The ischemia/reperfusion + saline treatment vehicle group (n=12) received i.p the same volume of vehicle (saline). Control group: Sham surgery was performed without occlusion of the carotid arteries and rats received i.p. injection of the same volume of saline. Animals were sacrificed 6 and 24hrs after ischemia (n=6 per group and per time point). At each time point, their brains were removed and were frozen in liquid nitrogen and stored at -80°C until further use.

**Measurement of SOD:**

Superoxide dismutase (SOD) activity in brain homogenate was measured through the inhibition of nitroblue tetrazolium (NBT) reduction by O$_2$\textsuperscript{-} generated by the xanthine/xanthine oxidase system. One SOD activity unit was defined as the enzyme amount causing 50% inhibition in 1mL reaction solution per milligram tissue protein and the result was expressed as U/mg protein \[41\].

**Caspase-3 activity assay:**

For measurement of caspase-3 activity, brain tissue samples were homogenized in 1.5mL of PBS, assayed with the CPP32/caspase-3 colorimetric protease assay kit (Chemicon International, Inc), and read at 405nm in a microtitr reader. Activity was standardized to total protein concentration determined with a bichinchoninic acid (BCA) total protein detection kit (Pierce Chemical Co) \[42\].

**Measurement of HSP-70:**

HSP70 protein expression in brain tissue was detected using a commercial HSP70 ELISA kit (Stressgen Bioreagents, Ann Arbor, MI). The protein was isolated from brain tissue homogenate by using protein extraction reagent. According to the assay procedure supplemented with the kit, HSP70
Detection of NF-κB, UCP-1, ghrelin receptor & beclin gene expression by Real-time RT-PCR:

The samples of brain tissue were dissected immediately after decapitation and frozen in liquid nitrogen. Total-RNA was extracted from tissue samples using Trizol reagent (Invitrogen) according to standard protocol as described previously (Brinkhof et al., 2006). The standard amount of total RNA (500ng) was used to synthesise the first-strand cDNA (High Capacity RNA-to-cDNA kit, Applied Biosystems, Foster City, CA). RT-PCR amplification mixtures (25 µl contained 1 µl template cDNA, SYBER Green master mix buffer (Quanti-Tect, Qiagen, Hilden) and 400nM (10 pmol/reaction) forward and reverse primer (Table 1). Reactions were run on step one plus Real-Time PCR detector (Applied Biosystems). The results were analysed by SDS software vs. 2.3 (Applied Biosystems). The expression of genes of interest was normalized to the housekeeper gene beta-actin and calculated using AA Ct method [44].

Table (1): Sequence of the primers used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>NFK-κB</td>
<td>Forward: 5´- GCTTACGGTGTTGGATTG-CAT-3´</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5´- TTATGGTGCCATGGGT-GATG-3´</td>
</tr>
<tr>
<td>UCP-2</td>
<td>Forward: 5´- AATGACATCTATGGCAAC-CCC-3´</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5´- AAGAACCACCATCTTCT-TG-3´</td>
</tr>
<tr>
<td>Ghrelin receptor</td>
<td>Forward: 5´- CAAGAATGGCGACCGTG-GTGA-3´</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5´- GGTGTGACTCGTGCAGC-CATC-3´</td>
</tr>
<tr>
<td>Beclin</td>
<td>Forward: 5´- TTCAAGATCCTTGGACCGAC-AAGTGAG-3´</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5´- AGACCATCCTTGGCGAGGTT-3´</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: 5´- ATCATGTTGAGACCT-TCAACC</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5´- TAGCTTCTTCCAGG-GAGG-3´</td>
</tr>
</tbody>
</table>

Statistical analysis:

Data were statistically analyzed using the statistical package for social sciences (SPSS 16.0 software). The results were expressed as the mean values ± standard deviation of six animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA). Subsequent multiple comparisons between the different groups were analyzed by post hoc test: Bonferroni in normally distributed quantitative variables and correlation analysis was performed using pearson test. Values at p<0.05 were considered significant.

Results

Effect of ghrelin administration on NF-κB:

There was significant increase in NF-κB mRNA at 6 & 24hrs of I/R in comparison to control groups and there was more significant increase in NF-κB in I/R model at 24hrs than at 6hrs (p<0.05). In I/R model with ghrelin administration NF-κB was significantly decreased at 6hrs and 24hr when compared to I/R model (p<0.05). Comparison of NF-κB values in the two groups of I/R model with ghrelin administration at 6 & 24hrs there was no significant change between them. Administration of ghrelin prevents the increase of NF-κB mRNA in I/R of the brain as there was no significant change in NF-κB mRNA values between I/R model with ghrelin administration and the corresponding control values (p>0.05) (Fig. 1).

Effect of ghrelin administration on SOD:

SOD value was significantly decreased in I/R in comparison to control groups and there was more decrease in SOD at 24hrs than at 6hrs but insignificant. In I/R model with ghrelin administration there was significant increase in SOD at 6hrs and 24hr in comparison to their values in I/R model (p<0.05). While SOD values in I/R model with ghrelin administration was significantly less than the corresponding control values (p<0.05). There was insignificant decrease in SOD values at 24hrs than at 6hrs in I/R model with ghrelin administration (p>0.05) (Fig. 2).

Effect of ghrelin administration on caspase3:

I/R caused significant increase in caspase3 after 6 & 24hrs in comparison to control groups (p<0.05) and there was more significant increase in caspase3 in I/R model at 24hrs than at 6hrs. It was significantly decreased at 6hrs and 24hr in I/R model with ghrelin administration when compared to I/R model. The caspase3 values in I/R model with ghrelin administration was insignificant in comparison to the corresponding control values.
There was no significant change in caspase3 values in the two groups of I/R model with ghrelin administration at 6 & 24hrs (Fig. 3).

**Effect of ghrelin administration on beclin:**

In I/R brain model beclin mRNA (marker of autophagy) was significantly decreased in comparison to its corresponding control values \((p<0.05)\). In I/R model with ghrelin administration geng expression of beclin was significantly higher than its values in I/R model \((p<0.05)\). At 24hrs from administration of ghrelin beclin mRNA returned to corresponding control value as there is no significant change when compared to each other \((p>0.05)\) (Fig. 4).

**Effect of ghrelin administration on HSP70:**

There was significant increase in HSP70 in 6 & 24hrs of I/R in comparison to its in value control groups \((p<0.05)\) and there was more increase in HSP70 in I/R model at 24hrs than at 6hrs but insignificant \((p>0.05)\). In I/R model with ghrelin administration HSP70 was significantly decreased at 6hrs and 24hr when compared to I/R model. Comparison of HSP70 values in the two groups of I/R model with ghrelin administration at 6 & 24hrs there was no significant change between them. The HSP70 is significantly higher in ghrelin treated rats than the control values \((p<0.05)\) (Fig. 5).

**Effect of ghrelin administration on UCP2:**

I/R of the brain caused significant increase in UCP2 when compared to controls, there was more significant increase after 24hrs than after 6hrs from ischemia \((p<0.05)\). In I/R model with ghrelin administration there was insignificant increase in UCP2 at 6hrs and 24hr in comparison to their control values \((p>0.05)\) but significantly lower than its values at the corresponding time in I/R model \((p<0.05)\) (Fig. 6).

**Effect of ghrelin administration on Gh receptor:**

Ghrelin receptors mRNA (Gh receptor) was significantly decreased by I/R of the brain at the two measured time when compared to corresponding control levels \((p<0.05)\). In I/R model with ghrelin administration the gene expression of receptor was significantly increased when compared to their values in I/R model but still significantly lower than the corresponding control levels \((p<0.05)\). Also with ghrelin administration there was significant increase in the receptors mRNA at 24hrs when compared to its value at 6hrs \((p<0.05)\) (Fig. 7).

Table (2): Values of the parameters in the six studied groups are expressed as Means±SD (n=6).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control 6 h</th>
<th>Control 24 h</th>
<th>I/R 6 h</th>
<th>I/R 24 h</th>
<th>I/R + Gherlin 6 h</th>
<th>I/R + Gherlin 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-icB (ng/mg ptn)</td>
<td>0.19±0.04</td>
<td>0.198±0.01</td>
<td>0.67±0.16*</td>
<td>1.22±0.25***</td>
<td>0.33±0.05 ab</td>
<td>0.37±0.09 ab</td>
</tr>
<tr>
<td>SOD (U/mg ptn)</td>
<td>2.48±0.42</td>
<td>2.483±0.42</td>
<td>0.66±0.13*</td>
<td>0.43±0.098**</td>
<td>1.53±0.36 ab</td>
<td>1.22±0.23 **ab</td>
</tr>
<tr>
<td>Caspase3 (ng/mg ptn)</td>
<td>0.5±0.096</td>
<td>0.518±0.09</td>
<td>2.48±0.55*</td>
<td>4.88±0.63**a</td>
<td>0.72±0.15 ab</td>
<td>0.99±0.14 ab</td>
</tr>
<tr>
<td>Beclin (pg/mg ptn)</td>
<td>10.03±1.55</td>
<td>9.67±1.18</td>
<td>5.12±0.05*</td>
<td>4.35±0.95**</td>
<td>6.72±1.54 b</td>
<td>8.68±0.98 ab</td>
</tr>
<tr>
<td>HSP-70 (pg/mg ptn)</td>
<td>2.22±0.45</td>
<td>2.35±0.26</td>
<td>11.88±1.94*</td>
<td>13.16±2.32***</td>
<td>6.37±1.44 ab</td>
<td>5.87±1.39 **ab</td>
</tr>
<tr>
<td>UCP2</td>
<td>0.72±0.13</td>
<td>0.737±0.098</td>
<td>2.3±0.46*</td>
<td>5.2±1.22***a</td>
<td>0.90±0.11 ab</td>
<td>1.28±0.32 ab</td>
</tr>
<tr>
<td>Ghrelin receptor</td>
<td>1.70±0.26</td>
<td>1.76±0.18</td>
<td>0.55±0.12*</td>
<td>0.4±0.07**</td>
<td>0.73±0.16* b</td>
<td>1.21±0.21 **abc</td>
</tr>
</tbody>
</table>

* Significant difference from 6hr control group.
** Significant difference from 24hr control group.

a: Significant difference from I/R 6hr group.
b: Significant difference from I/R 24hr group.
c: Significant difference from I/R 6hr + Gherlin group.
Significant difference at \(p<0.05\).
Discussion

Oxidative stress and delayed inflammation are critical factors facilitating neuronal death after cerebral ischemia-reperfusion injury [45]. It has been established that ROS production is increased after cerebral ischemia and reperfusion [46,47] and such increases initiate expression of inflammatory cytokines [48]. These, in turn, stimulate innate inflammation to generate more ROS, creating a positive feedback mechanism [48]. The previous studies are in line with results of the present study which revealed that I/R-induced brain injury, evidenced by decrease in SOD activity indicating increase in ROS. Furthermore, mRNA of NF-κB which plays a pivotal role in inflammatory process was increased in the brain tissue of the I/R group [49], our results showed that ghrelin administration diminished I/R-induced oxidative stress as SOD activity in brain tissue was significantly increased versus I/R rats. Ghrelin has been shown to reduce oxidative stress in the stomach after ischemia [50], to inhibit hydrogen peroxide-induced apoptotic cell death of oligodendrocytes [51] and to inhibit oxygen-glucose deprivation (OGD)-induced cell death by suppression of ROS generation [27]. Also, Obay et al., [52] found that ghrelin pretreatment diminished pentylenetetrazole-induced oxidative stress and prevented the decrease in antioxidant enzyme activities in brain tissue, and thus may reduce neuronal death in the brain during seizures.

In the present study we found that ghrelin treatment prevented the increase in NF-κB, indicating that ghrelin, has anti-inflammatory effect, protected the brain against I/R-induced injury. In agreement with our results, He et al., [53] reported
that acylated ghrelin decreased phosphorylated NF-xB p65 and inhibit the release of inflammatory cytokines in human monocyte-derived (THP-1) macrophages which are stimulated by palmitic acid in a dose-dependent fashion. Also Wu et al., [54] found that ghrelin can be developed as a novel treatment for severe sepsis-induced acute lung injury (ALI) and improved survival in sepsis through inhibition of NF-kappa B and helps control at least some inflammatory diseases.

Caspase-3 has been identified as a key mediator of apoptosis in animal models of ischemic stroke. Asahi and coworkers [58] demonstrated upregulation of caspase-3 mRNA in rat brain 1 hour after the onset of focal ischemia. In addition, Namura and associates [56] detected caspase-3 and its cleavage products in mouse brain during early reperfusion after 2-hour middle cerebral artery (MCA) occlusion. Importantly, comparable observations have been extended to ischemic human brain tissue in that caspase-3 was upregulated after ischemia [57]. The neuroprotective effects of ghrelin in the middle cerebral artery (focal) and four vessel occlusion (forebrain) models of ischemia reperfusion, are mediated by suppressing the increase in the pro-apoptotic gene, Par-4, associated with the ischemia. Ghrelin also improves the Bcl2/BAX ratio and inhibits cytochrome c release and caspase-3 activation [26,58,59]. The previous studies are in agreement with our results which showed that I/R caused increase in caspase3 in the brain tissue after 6 and the increase was more significant at 24hrs than at 6hrs. Ghrelin administration prevented this increase as the caspase3 values in I/R model with ghrelin administration was the same as the corresponding control values measured at 6h and 24h. Also the antioxidant effect of ghrelin prevents apoptosis as contribute to transduction of apoptotic signals ROS [60].

Beclin 1 has a key role in the initiation of autophagy, a process of self-digestion in which cytoplasmic constituents are sequestered and targeted for lysosomal degradation [61]. Beclin 1 represents an important component of the autophagic machinery. It interacts with proteins that positively regulate autophagy [62]. In the present study beclin1mRNA was reduced in the rat brain I/R and it was increased in I/R rats administrated ghrelin. At 24hrs from administration of ghrelin in I/R rat brain model beclin1 returned to corresponding control value. These results suggested that one of the neuroprotective mechanisms of ghrelin is stimulation of autophagy. Tong et al., [34] found that ghrelin treatment significantly attenuated CoCl(2)-induced hypoxic injury in cardiac H9c2 cells by increasing autophagy levels and revealed that inhibiting autophagy using 3-methyladenine or AMPK pathway with compound C almost abrogated the induction of ghrelin in autophagy. This was associated with a decrease in cell viability. It has been found that activation of autophagy by rapamycin and simvastatin, decreased neuronal apoptosis and ameliorated early brain injury after subarachnoid hemorrhage model in rats [63]. Also activation of autophagy during cerebral ischemia by nicotinamide phosphoribosyltransferase promotes neuronal survival [64].

In the present study HSP70 was showed marked increase in brain tissue of I/R model induced by oxidative stress. While ghrelin administration caused moderate increase in HSP70 at 6h and 24h in rat brain I/R model, this is in accordance with the study of Qin et al., [65] who revealed that ghrelin increased the expression of HSP70 in spinal cord I/R at 24 and 48hrs. HSP70 may have anti-inflammatory, cytoprotective and an anti-apoptotic actions [66,67,68].

UCP2 message has been shown to be up-regulated in the CNS by stress signals such as kainate administration or ischemia, and overexpression of UCP2 has been reported to be neuroprotective against oxidative stress in vivo and in vitro [69]. Increased UCP2 expression was observed in mitochondria of hippocampal CA1 2-24h after transient global ischemia TGI/reperfusion, with maximal expression levels at 6-18h [70,71]. The previous studies are in agreement with the results of the present study as I/R of the brain caused increase in UCP2 mRNA with maximal expression levels at 24h. In I/R model with ghrelin administration there was insignificant increase in UCP2 at 6hrs and 24hr in comparison to their control values, while Liu et al., demonstrated upregulation of hippocampal UCP2 mRNA in 4vessels occlusion I/R model on day 3 of ghrelin administration. It is likely that this protocol led to a more persistent upregulation of UCP2 than would have been induced by a single injection of ghrelin or a single preconditioning ischemia [72].

In the present study we found that I/R-induced injury of rat brain caused significant decrease in ghrelin receptor mRNA while ghrelin administration reversed the downregulation of GHSR-1 mRNA in rat brain at 24 h after I/R injury. In agreement with our results it has been found that the expression of ghrelin’s receptor Gh receptor in rat cerebral cortex were obviously decreased by ischemia/reperfusion injury and increased by ghrelin (i.v.) [73]. Qin et al., [65] also found that the ghrelin receptor (GHSR-1a) gene expression in the spinal
cord decreases in presence of I/R-induced injury, and ghrelin reverses the down regulation of GHSR-1a mRNA and protein in rat spinal cords at 24h after I/R [65]. The GHS-R1a has considerable constitutive activity in the absence of the ligand and may significantly contribute to limiting apoptosis after ischemia even when ghrelin is low [74].

Conclusion:
Our results indicate that ghrelin protected rat brain against I/R injury and its early administration can improve neuronal cell survival in animal models of cerebral ischemia. The possible mechanisms of action including anti-apoptotic, anti-inflammatory, anti-oxidant effects of ghrelin. Also our results showed that ghrelin induced autophagy and increased gene expression of its receptors. We suggest that ghrelin treatment may be a useful intervention after stroke in the clinic.

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