

## Cystatin C and neuropeptid Y levels in brain tissues after experimental subarachnoid hemorrhage

Şerefden Açıkgöz<sup>1</sup>✉, Murat Can<sup>1</sup>, Berrak Güven<sup>1</sup>, Nurullah Edebalı<sup>2</sup>, Figen Barut<sup>3</sup>, Çağatay Büyükuysal<sup>4</sup>, İshak Özel Tekin<sup>5</sup> and Bektaş Açıkgöz<sup>2</sup>

<sup>1</sup>Department of Biochemistry, <sup>2</sup>Department Neurosurgery, <sup>3</sup>Department of Pathology, <sup>4</sup>Department of Biostatistics, <sup>5</sup>Department of Immunology Faculty of Medicine, Bülent Ecevit University (Formerly, Zonguldak Karaelmas University), Zonguldak, Turkey

The aim of this study was to investigate the changes in the levels of cystatin C, which protects neurodegeneration in the central nervous system with the inhibition of cysteine protease and by inducing autophagy in the pathogenesis of cerebral vasospasm and levels of vasoconstrictive neuropeptid Y (NPY) in the brain tissue homogenates of rat model of subarachnoid hemorrhage (SAH). Three experimental groups were used: Day 2 and Day 7 groups after SAH, and also a control group. There were seven Wistar albino rats in each group. SAH was accomplished by transclival basilar artery puncture. Rat cystatin C, rat NPY were determined with ELISA in brain tissue homogenates. Day 2 group showed significantly enhanced cystatin C values in comparison with the control group ( $P=0.048$ ). NPY levels between the Day 2 and Day 7 groups and the control groups were not significantly different ( $P=0.315$ ). In histopathological examination, there was less neuronal loss in the Day 2 group than in the Day 7 group. Regarding our results, it would be more valuable to measure NPY levels in specific brain areas. The increased cystatin C levels on the second day after SAH is probably a pathophysiologic mechanism to organize protease activity.

**Key words:** cystatin C, neuropeptid Y, experimental subarachnoid hemorrhage

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### INTRODUCTION

Subarachnoid hemorrhage (SAH) is a disease causing high mortality and morbidity. Besides the hemorrhage, also the delayed ischemic deficit or the delayed cerebral vasospasm are mainly accused as the responsible factors in the death or neurologic deficits seen after SAH. Studies in recent years also underline another factor, the early brain injury which is associated with these problems (Cahill & Zhang, 2009; Nishizawa, 2013). In the pathophysiology of early brain injury after SAH, apoptotic pathways, inflammatory pathways, and ischemic pathways were accused (Cahill & Zhang, 2009).

Neuropeptid Y (NPY) is a vasoconstrictive neuropeptid expressed in human brain (Schebesch *et al.*, 2011). It is colocalized in sympathetic fibers with noradrenaline and is mostly localized in the superior cervical ganglion as a neuropeptide taking part in the regulation of cerebral blood flow (Juul *et al.*, 1990).

There were studies that showed increased NPY activity in CSF of SAH patients (Suzuki *et al.*, 1989), and af-

ter experimental SAH (Abel *et al.*, 1988). In some studies, no change was seen in CSF levels after experimental SAH (Pluta *et al.*, 1992).

Some experimental studies examined the vasoconstrictive effect of NPY (Abel *et al.*, 1988; Uneyama *et al.*, 1995) and in some clinical studies on SAH patients, the relation between the NPY levels in CSF and vasoconstriction (Schebesch *et al.*, 2011; Juul *et al.*, 1990;) and cognitive functions (Uski *et al.*, 2000) were examined.

Cystatin C is a sistein protease inhibitor, which takes part in the regulation of a local inflammation (Umegae *et al.*, 2008). Most of the cystatin C is produced by nucleated cells and then, after glomerular filtration, it is catabolized by renal tubular cells (Grubb, 1992).

There were increases in serum cystatin C levels after acute cerebral stroke, as well as a correlation between cystatin C levels, hemorrhage volume and infarction size were demonstrated (Xiao *et al.*, 2012).

Cystatin C induces autophagy in vivo as a protective mechanism after brain injury and in neurodegenerative disorders (Gauthier *et al.*, 2011). In the literature, there were studies demonstrating increases in autophagy after SAH (Lee *et al.*, 2009; Wang *et al.*, 2012). Liu and coworkers (Liu *et al.*, 2013) demonstrated an increase of the autophagy proteins in the wall of basilar artery after cystatin C-treatment in experimental SAH. Cystatin C-treatment improved learning deficits (Liu *et al.*, 2014) induced by SAH.

In our literature review, we failed to find any earlier report that has measured cystatin C levels in brain tissues directly after experimental SAH.

It seemed reasonable to measure vasoconstrictive NPY and neuroprotective cystatin C in the same brain tissues simultaneously. Therefore, in this study, we investigated NPY and cystatin C levels in brain tissue homogenates obtained from rats after experimental SAH on Days 2 and 7, and compared with normal brain tissues. Histopathological examinations of the tissues were also carried out.

### MATERIAL METHODS

The study protocol was approved by the Bülent Ecevit University Animal Ethics Committee.

**Subjects.** The animal study was performed at the Experimental Surgery, Research and Animal Laboratory of Bülent Ecevit University, Faculty of Medicine, Zonguldak.

✉ e-mail: serefdenacikgoz@yahoo.com

**Abbreviations:** SAH, subarachnoid hemorrhage; NPY, neuropeptid Y; CSF, cerebrospinal fluid

21 male adult Wistar albino rats, weight 200–300 g, were included in the study. All rats were kept at 22–25°C with appropriate humidity, on a 12/12 h light/dark cycle, and were given fluids and food ad libitum. The rats were divided into three groups as follows: Day 2 after SAH (n=7), Day 7 after SAH (n=7) and controls (n=7).

**Surgical procedure.** Rats were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Experimental SAH was created with a technique similar to that described by Barry and coworkers (1979). Briefly, through a midline cervical incision in the supine position, using an operating microscope (Takagi OM-5, Japan) the clivus was exposed using the anterior parapharyngeal approach. A bony window in front of the basilar artery was created using large bore needles with the immaculate care not to open the preoptine cistern. A suture needle with an outer diameter of 75µm (Ethicon, Scotland, UK) was inserted into the basilar artery. Withdrawal of the needle caused an extensive hemorrhage into the subarachnoid space with an even distribution up to the olfactory area. The rats were kept alive for 2 and 7 days after SAH under appropriate conditions.

**Laboratory tests.** After craniotomy, brain tissues were extracted. Samples were taken from the right cerebral parietal hemisphere and immediately frozen at –80°C for analysis at a later date. Tissue samples from each rat brain were homogenized at 1/10 rate with a lysis buffer containing 50 mM Tris-HCL (pH=7.5), 150 mMol NaCl, 1% Triton X-100 (Koike *et al.*, 2013), by using a homogenizer (Ultra Turrax IKA T18 Basic). The levels of cystatin C, neuropeptid Y, total protein were measured in supernatant of the brain tissue homogenates following centrifugation, and the results were calculated as per mg tissue total protein.

Levels of cystatin C in rat brain tissue homogenates were determined by using the rat cystatin C ELISA kit (ICL Catalog no. E-25CYS, USA) with the double antibody sandwich method (briefly formed Antibody-cystatin C-HRP/Cystatin C/Antibody-cystatin C complex which catalyzes a color development reaction) in EPSON LX-300 ELISA device (BIO-TEC Instruments, Winooski, USA).

Levels of NPY in rat brain tissue homogenates were determined using the rat NPY ELISA kit (RayBio Catalog no. EIA-NPY-1, CHINA) with the principle of competitive Enzyme Immunoassay method (briefly both biotinylated NPY and NPY in targeted samples interact competitively with the NPY antibody, then unbound biotinylated NPY interacts with streptavidin HRP which catalyzes a color development reaction) in EPSON LX-300 ELISA device (BIO-TEC Instruments, Winooski, USA).

Tissue total protein levels using the colorimetry method (Siemens Healthcare Diagnostics, Tarrytown, NY, USA, C 2008-04) were assayed with Advia 2400 autoanalyzer (Siemens, Tarrytown, USA). The principle of the method is to measure the changes in absorption caused

by the complex of protein with pirogallol red-molibdate at acidic pH.

**Histopathologic Evaluation.** Tissue specimens from the brain were fixed in 10% neutral formalin solution and subjected to the routine processing for histopathologic examination. A pathologist microscopically examined the hematoxylin&eosin (H&E)-stained sections in blinded fashion.

**Statistical analysis.** The Statistical Package for Social Sciences 19.0 (SPSS Inc, Chicago, IL, USA) was used in all data analyses. The results were expressed as median, minimum-maximum. The normality distribution of the variables was tested using the Shapiro Wilk test. For the variables without normal distribution, Mann Whitney U-test was used for the two group comparisons.

Correlation analysis was performed with the Spearman correlation test. *P*-values less than 0.05 were considered statistically significant.

## RESULTS

### Biochemical findings

Day 2 group showed significantly enhanced cystatin C values (median (min-max)) (502 (434–573) ng/mg protein) compared with the control group (436 (319–486) ng/mg protein) (*P*=0.048).

Day 7 cystatin C values (448 (418–520) ng/mg protein) did not differ significantly from those in the control and Day 2 groups (Table 1).

NPY levels between Day 2 (59 (47–67) ng/mg protein), Day 7 (55 (47–61) ng/mg protein) and the control groups (63.5 (53–74) ng/mg protein) did not differ significantly (*P*=0.315) (Table 1). No correlation was found between the tissue cystatin C and NPY levels (*P*=0.491).

### Histopathologic findings

Examination of the brain sections from cerebral cortex regions with H&E staining, revealed healthy and damaged neurons. Typical appearance of healthy or intact neuron was presented with eosinophilic cytoplasm, prominent nucleus and intact nucleolus. Damaged neurons following SAH with irreversible cell damage were observed as shrinkage of the nucleus and vacuolization of the cytoplasm. When the neuronal damage of the three groups were evaluated separately, in the H&E sections, (A) in the control group about 2–5%, (B) in Day 2 about 30–40% and (C) in Day 7 about 60–70% of the neurons were damaged, respectively (Fig. 1).

## DISCUSSION

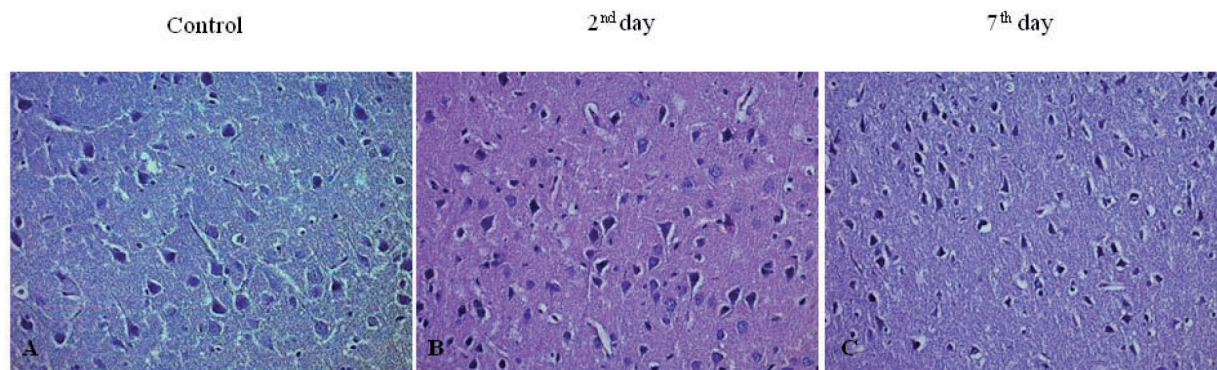
### Cystatin C

In some studies the role of cystatin C in regeneration/degeneration of neurons were investigated and the po-

**Table 1.** Levels of cystatin C and NPY in the brain tissue homogenates in SAH and normal rats

	2 <sup>nd</sup> day group Median (min-max) n=7	7 <sup>th</sup> day group Median (min-max) n=7	Control group Median (min-max) n=7	<i>P</i> value
Cystatin C (ng/mg protein)	502 (434–573)*	448 (418–520)	436 (319–486)	<i>P</i> =0.048
Neuropeptid y (ng/mg protein)	59 (47–67)	55 (47–61)	63,5 (53–74)	<i>P</i> =0.315

\*Difference from control



**Figure 1. Images of the tissue sections stained with hematoxylin and eosin from cerebral cortex region.** (A) control, (B) day 2, (C) day 7.

tential neuroprotective role of cystatin C was underlined (Miyake *et al.*, 1996; Nishio *et al.*, 2000; Palm *et al.*, 1995).

In the literature, there were studies demonstrating the increases of autophagy (Lee *et al.*, 2009; Wang *et al.*, 2012) and the relationship between autophagy and cystatin C (Liu *et al.*, 2013) after SAH. Lee and associates demonstrated increases in autophagy on day 1 in neurons, after experimental SAH (Lee *et al.*, 2009). Wang *et al.* demonstrated increased autophagy and autophagosome biomarkers on day 1 after experimental SAH and they underlined the protective role of autophagy in the early brain injury (Wang *et al.*, 2012). Liu *et al.* showed the increases in autophagy proteins at the basilar artery wall in 48 hours after SAH. They observed healing in the inner basilar artery caliber and basilar artery wall thickness after injection of cystatin C to cisterna magna. They underlined the possible restorative role of induced autophagy with cystatin C-treatment in the prevention of cerebral vasospasm (Liu *et al.*, 2013).

Liu and coworkers (Liu *et al.*, 2014) reported that learning deficits induced by SAH, were markedly improved after the cystatin C treatment. In conclusion, pre-SAH cystatin C administration may attenuate early brain injury and neurobehavioral dysfunction in that SAH model, possibly through activating the autophagy pathway.

In our study, cystatin C levels were found significantly increased in Day 2 group compared with the control group in brain tissue homogenates. The comparison between Day 7 and the control groups revealed nonsignificant changes (Table 1). In histopathological examinations, neural loss on Day 2 was less compared with Day 7 (Fig. 1). These results suggest the probable neuroprotective effect of the cystatin C.

### Neuropeptid Y

We did not observe significant changes in NPY levels in rat brain homogenates in Day 2 and Day 7 groups after SAH, compared with the control group (Table 1).

NPY was found effective in the regulation of vessel diameter and cerebral blood flow in SAH patients (Juil *et al.*, 1990; Schebesch *et al.*, 2011). In another study, NPY concentrations of CSF were found high after aneurysmal SAH (Suzuki *et al.*, 1989).

Previously, increased CSF and serum NPY levels in patients with cerebral vasospasm after SAH than in the patients without vasospasm were demonstrated (Schebesch *et al.*, 2011). Schebesch and coworkers (Schebesch *et al.*, 2013) have noted NPY as a valuable biomarker in the detection of cerebral vasospasm and

ischemia in SAH patients with aneurysm rupture. Abel and coworkers (Abel *et al.*, 1988) observed in rabbits increased CSF NPY on day 3 after SAH, and their *in vitro* studies revealed that NPY may potentiate the vasoconstrictor effect of norepinephrine, which contributes to the development of cerebral vasospasm.

There were previous reports showing regional differences of NPY immunoreactivity in the brain tissues.

The distribution of NPY immunoreactivity in the rat brain shows similarity to humans, but they are not identical. NPY immunoreactivity was the highest in the rat periaqueductal grey, nucleus accumbens, hypothalamus, septum and amygdala; lower amounts of NPY immunoreactivity were found in the basal ganglia, globus pallidus, hippocampus and cortex (Allen *et al.*, 1983; Wettstein *et al.*, 1995). Lin and associates found high NPY mRNA at corpus striatum, cerebral cortex and hypothalamus of rats after SAH compared with normal tissues (Lin *et al.*, 2002).

Our measurements were carried out in one area only (right parietal area), what narrows down our study.

In conclusion, in the view of the present study and literature survey, we think it will be more reasonable to measure NPY in different parts of the brain simultaneously.

Cystatin C was found significantly increased on the Day 2 following SAH. Histopathological examinations revealed less neuronal loss on the Day 2 post SAH than on the Day 7. Increased cystatin C levels in the rat brain tissues, on Day 2 after SAH may play a potential role in the regulation of protease activity, which is supposed to be a restorative process after SAH.

### Conflict of Interest

The authors declare no conflict of interest

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