Biochemical changes representing oxidative stress on brain tissue due to intraabdominal hypertension in a rat model

Hamit S. Karabekir1, Feyza K. Guzey2, Canan Balci3, Yesim Guvenc4, Ece Onur4, Gokhan Akbulut5, Mustafa Serteser6

Abstract

Introduction: Intraabdominal hypertension affects the central nervous system in addition to respiratory, renal and cardiovascular systems. This effect that, investigated in detail by clinical and experimental studies, is due to the increase of intracranial pressure and decrease of cerebral perfusion pressure caused by the increase of intrathoracic pressure and increase of pressure of great veins. However, no study has been found on biochemical changes on central nervous tissue due to intraabdominal hypertension.

Material and methods: Thirty rats were divided into three groups containing 10 animals: sham group, study group I and study group II. In group I, intraabdominal pressure was elevated to 20 mmHg, and in group II, it was elevated to 30 mmHg for 60 minutes. Intracranial pressures (ICP) in all animals were monitored. Values of biochemical parameters including thiobarbituric acid, nitrite oxide, xanthine oxidase, protein carbonyls and protein sulfhydryl in cortical, subcortical, cerebellar and spinal cord tissues were compared with the corresponding values in sham rats.

Results: Thiobarbituric acid (0.58±0.8 and 0.76±0.04 vs. 0.23±0.03, p<0.05 and p<0.001), nitrite oxide (3.11±0.10 and 8.46±0.54 vs. 1.52±0.18, p<0.05 and p<0.001), xanthine oxidase (1.55±0.11 and 3.01±0.25 vs. 0.32±0.09, p<0.001) and carbonyl levels (1.41±0.01 and 1.69±0.01 vs. 1.22±0.03, p<0.001) of the various central nervous system regions and ICP were increased. SH levels (92.60±2.50 and 74.60±3.80 vs. 139.20±4.72, p<0.001) were decreased after intraabdominal hypertension, and higher IAP generally caused more detrimental effects on these parameters. The levels of spinal cord and cerebellum samples were significantly worse in the study groups for most of the markers.

Conclusions: Intraabdominal hypertension may cause biochemical changes representing oxidative stress on various regions of central nervous system even 60 minutes after increase of intraabdominal pressure, and higher IAH causes more detrimental effects. Most prominent effects were seen in spinal cord and cerebellar tissue suggesting that compression of lumbar vertebral venous pressure might have a role in addition to increase of ICP due to increase of pressure of great veins caused by increase of intrathoracic pressure.

Key words: abdominal compartment syndrome, cerebral perfusion pressure, intraabdominal hypertension, intracranial pressure, oxidative stress.
Introduction

Abdominal compartment syndrome (ACS) is a clinical entity compromising a series of pathophysiologic changes induced by intraabdominal pressure (IAP) elevation that involves both intra- and extrabdominal organs and systems. The results of recent studies clearly demonstrate that ACS does not represent a rare clinical entity but a critical complication in the very early period after trauma in certain subgroups, mostly in multiply injured patients with abdominal or pelvic trauma [1-3], and in the patients with some medical diseases and conditions such as acute pancreatitis or massive fluid replacement [2, 4-6]. Systemic effects are due to increase of intrathoracic pressure (ITP), of pressure in great veins because of elevation of the diaphragm, and of intracranial pressure (ICP) [2, 5, 7, 8].

Destruction of brain tissue in trauma patients occurs in two phases: the primary injury, which involves direct tissue damage at the time of the inciting event, and the subsequent secondary injury, which occurs due to ischemia, causing calcium ion influx and free radical accumulation. Primary injury is considered irreversible, and therefore therapy is directed toward minimizing the secondary brain injury by optimizing cerebral perfusion pressure (CPP) and cerebral blood flow and by aggressive treatment of intracranial hypertension. The relationship between intracranial, intrathoracic, and intra-abdominal compartment pressures has been described in both animal models and human series [9-13]. Measures that reduce IAP reduce ICP [14]. It is important that trauma surgeons, neurosurgeons, and intensivists be aware of this association, because up to 50% of patients with major abdominal trauma also have a significant head injury [15].

No study has been found on biochemical changes of neural tissue on ICP elevations due to IAH. We planned a study evaluating changes of nitric oxide (NO) level, xanthine oxidase (XO) activity, thiobarbituric acid (TBARS) level, an indicator of malonyldialdehyde (MDA), protein carbonyls and protein sulfhydryl (SH) levels of various neural tissues due to IAH in a rat model to show brain oxidant-antioxidant status.

Material and methods

Subjects

Thirty adult females, specific pathogen free Sprague Dawley rats (200-220 g) purchased from Selcuk University, Experimental Research Laboratories (Konya, Turkey) were included into this study. Animals were isolated from male rats and were housed in standard laboratory cages. They were allowed free access to food and water till 12 hours before the surgical procedure. All procedures mentioned were approved by the local ethics authority. The study protocol was designed in accordance with the 1996 revised form of The Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Unnecessary animal suffering was avoided throughout the study.

Thirty rats randomly assigned into one of three experimental groups: sham group (n=10), study group I (n=10), and study group II (n=10). In sham group, parameters were measured without increase of intraabdominal pressure (IAP); in group I, they were measured after increase of IAP to 20 mmHg for 60 minutes, and in group II, they were measured after increase of IAP to 30 mmHg for 60 minutes.

Surgical procedure and raising of intraabdominal pressure

The rats in all groups were anesthetized with ketamine hydrochloride (100 mg/kg, im, Ketalar amp, EIP, Istanbul, Turkey). The rats were first fixed within a head holder. After the area was sterilized with povidone iodine solution, a midline incision on the skin was made up to the neck area in order to permit approach to the cisterna magna. The atlantooccipital membrane was punctured using a 23-gauge needle; the appearance of clear cerebrospinal fluid (CSF) indicated good entrance. The needle was fixed in the cisterna magna space using cyanoacrylate. The needle was connected with a plastic serum tube and the tube was connected to a pressure transducer (Datex-Ohmeda Division, Instrumentarium Corp., Helsinki, Finland). After this procedure, the abdominal region was shaved and sterilized with povidone iodine solution. The SaO2 and HR were measured with pulse-oxymeter (Datex-Ohmeda Division, Instrumentarium Corp., Helsinki, Finland). A plastic tube, 5 mm in thickness, was prepared by having one end tightly secured to a serum physiologic bag and placed intraperitoneally in a manner to prevent leaking fluid in aseptic conditions. A linear mercury manometer was placed between the plastic tube and serum physiologic bag, and IAP was measured throughout the study (Figure 1). The IAP was maintained at that level for 60 minutes. At the end of this waiting period, the subjects were decapitated and brain and medulla spinalis were removed. Tissue samples were frozeed at -20°C until biochemical measurements were performed.

In the sham group, the rats were anesthetized in a same manner with the study groups, IAP was not elevated, and they were also decapitated and tissue samples were taken.

Tissue preparation

The tissue samples were washed three times with physiologic saline solution to remove any
contaminant. A portion of each tissue sample was placed in a 2 ml cold Tris-Cl (0.2 M, pH=7.5) buffer contained in glass tubes and homogenized for 3 min at 13 000 rpm (IKA T18 basic, Wilmington NC). After homogenization, the homogenates were subjected to centrifugation at 4000 rpm, +4°C for 30 minutes. The supernatants were removed after centrifugation and stored at –20°C for further biochemical analysis.

**Biochemical analysis**

If not stated otherwise, all chemicals were purchased from Sigma Chemical Co. (St. Lois, MO). Brain tissue thiobarbituric acid reactive substances (TBARS) levels, as an indicator of lipid peroxidation were determined according to the spectrophotometric method described by Okhawa et al. [16]. The results were expressed as nmol/mg protein. NO levels were determined by using a previously described method based on Griess reagent [17]. After deproteinization with Somogy [18] reagent to eliminate any interference, the sample was subjected to nitrite analysis by Griess reagent. In order to reduce the nitrate to nitrite, the sample was incubated with copper coated cadmium granules for 90 minutes. By using this incubated sample for nitrite analysis, the total NO levels (nitrate + nitrite) were calculated. The results were expressed as μmoles/g protein. XO activity of different brain regions were determined according to the method of Prajda and Weber [19]. The assay depends on the rate of the conversion of xanthine to uric acid by the enzyme XO. The results were expressed as U/g protein.

PC's levels were measured by using a method described by Levine et al. [20]. Results were expressed as mol carbonyl/mg tissue protein.

PSHs were measured spectrophotometrically using Ellman’s reagent, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), with the thiol-disulfide interchange reaction between DTNB and thiol providing the basis of the assay [21]. Results were expressed as μmol/mg tissue protein. Tissue proteins were evaluated by the method of Lowry [22].

**Statistical analysis**

Data were analysed by using SPSS (Statistical Package for the Social Science), version 10.0 for Windows 98 (Microsoft Corp.). Results were expressed as mean ±SEM (Standard Error of Mean). One-way analysis of variance (ANOVA) was used to determine the significance of any differences between groups. Statistical comparisons between groups were performed by nonparametric Mann-Whitney U test and the difference was considered to be significant when p<0.05. A Bonferroni correction was calculated for each group of comparisons after analysis of variance.

**Results**

**Brain tissue TBARS levels (Figure 2)**

Cortical tissue TBARS levels in group I and II were found to be higher than those in sham animals (p<0.05 and p<0.001 respectively). Cerebellar tissue levels were found to be higher only in the group II animals when compared to sham animals (p<0.05). Medulla spinalis levels were found to be significantly higher in group II animals than sham animals and group I (p<0.001). No statistically significant differences were found between different brain regions in both sham group and in group I. But medulla spinalis levels in group II were found to be higher than those of cortical tissue levels in the same group (p<0.001), (Table I).

![Figure 1](image1.png)

**Figure 1.** Basic diagram for increasing intraabdominal pressure by using serum physiologic. You can measure the pressure by a linear mercury manometer

![Figure 2](image2.png)

**Figure 2.** TBARS levels of the groups. Values are mean ±SEM nanomoles per milligram tissue protein: a) p<0.05 vs. sham cortex, b) p<0.001 vs. sham cortex, c) p<0.05 vs. sham cerebellum, d) p<0.001 vs. sham and group I medulla spinalis, vs. group II cortex.
Brain tissue NO levels (Figure 3)

Cerebellar tissue NO levels were found to be higher both in group I and II than sham group. The highest medulla spinalis NO levels were observed in group II animals (p<0.001). No statistically significant differences were found between different brain regions in NO levels in the sham group, but the highest NO levels were observed in medulla spinalis in group I and in cerebellum and medulla spinalis in group II (p<0.001), (Table II).

Brain tissue XO activity (Figure 4)

Cerebellar tissue XO levels were found to be higher both in group I and II than sham group. The highest medulla spinalis XO activities were observed in group II animals (17.43±0.83 U/g protein, p<0.001). No statistically significant differences were found between different brain regions in XO activities in sham group but the highest XO activities were observed in cerebellum and medulla spinalis in group I and II (Table III).

Table I. TBARS level of the groups, we remarked the statistically different values (p<0.05 and p<0.001); NS – no significant differences (nmol/mg protein)

<table>
<thead>
<tr>
<th>TBARS levels</th>
<th>Cortical</th>
<th>Subcortical</th>
<th>Cerebellar</th>
<th>Medulla spinalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.23±0.03</td>
<td>NS</td>
<td>0.37±0.04</td>
<td>0.49±0.04</td>
</tr>
<tr>
<td>Group I</td>
<td>0.58±0.08</td>
<td>NS</td>
<td>0.74±0.03</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>0.76±0.04</td>
<td>p&lt;0.001</td>
<td>0.78±0.05</td>
<td>1.33±0.13</td>
</tr>
</tbody>
</table>

Table II. NO levels; *p<0.05 between sham and group I; α – p<0.001 between sham and group I; π – p<0.001 between group I and group II; NS – no significant differences

<table>
<thead>
<tr>
<th>NO levels</th>
<th>Cortical</th>
<th>Subcortical</th>
<th>Cerebellar</th>
<th>Medulla spinalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1.52±0.18</td>
<td></td>
<td>1.62±0.21</td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>NS</td>
<td>NS</td>
<td>3.11±0.10*</td>
<td>6.64±0.48</td>
</tr>
<tr>
<td>Group II</td>
<td>NS</td>
<td>NS</td>
<td>8.46±0.54α π</td>
<td>8.82±0.27</td>
</tr>
</tbody>
</table>

Hamit S. Karabekir, Feyza K. Guzey, Canan Balci, Yesim Guvenc, Ece Onur, Gokhan Akbulut, Mustafa Serteser
Brain tissue SH levels (Figure 5)

Cortical tissue SH levels were found to be significantly decreased both in group I and II when compared to sham group. Subcortical SH levels did not reveal any significant change between sham group and group I but significantly decreased levels were observed in group II. On the other hand, SH levels in cerebellum and medulla spinalis in group II were found to be significantly decreased than sham group and group I. Only cortical levels were found to be significantly different from subcortical and cerebellar levels in sham group. In group I, medulla spinalis SH levels were found to be significantly lower than cortical tissue level. No statistically significant differences were observed between different brain regions in group II animals (Table IV).

Brain tissue protein carbonyl levels (Figure 6)

Protein carbonyl levels were found to be significantly higher in all brain regions in group II than sham group. Values are mean ±SEM mole carbonyl per milligram tissue protein: a) p<0.001 vs. sham cortex, b) p<0.001 vs. sham subcortex, c) p<0.001 vs. sham cerebellum, d) p<0.001 vs. sham medulla spinalis, e) p<0.001 vs. cortex and subcortex in the same group. Values are mean ±SEM mole carbonyl per milligram tissue protein: a) p<0.001 vs. group I cortex, b) p<0.001 vs. group I subcortex, c) p<0.001 vs. cortex and subcortex in the same group, d) p<0.05 vs. cortex in the same group.

Table III. XO levels; *Statistically difference between sham and group I and group II

<table>
<thead>
<tr>
<th>XO levels</th>
<th>Cortical</th>
<th>Subcortical</th>
<th>Cerebellar</th>
<th>Medulla spinalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>NS</td>
<td>NS</td>
<td>0.32±0.09</td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>NS</td>
<td>NS</td>
<td>1.55±0.11</td>
<td>5.88±0.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p&lt;0.001*</td>
<td>p&lt;0.001*</td>
</tr>
<tr>
<td>Group II</td>
<td>NS</td>
<td>NS</td>
<td>3.01±0.25</td>
<td>17.43±0.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p&lt;0.001*</td>
<td>p&lt;0.001*</td>
</tr>
</tbody>
</table>

Table IV. SH levels; *Statistically significant between sham and groups

<table>
<thead>
<tr>
<th>SH levels</th>
<th>Cortical</th>
<th>Subcortical</th>
<th>Cerebellar</th>
<th>Medulla spinalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>139.20±4.72</td>
<td>110.40±4.74</td>
<td>113.40±6.56</td>
<td>129.80±3.65</td>
</tr>
<tr>
<td>Group I</td>
<td>92.60±2.50</td>
<td>NS</td>
<td>91.00±3.58</td>
<td>120.00±4.33</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.001*</td>
<td></td>
<td>p&lt;0.001*</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>74.60±3.80</td>
<td>79.40±3.60</td>
<td>72.60±3.90</td>
<td>102.40±5.46</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.001*</td>
<td>p&lt;0.001*</td>
<td>p&lt;0.001*</td>
<td>p&lt;0.05*</td>
</tr>
</tbody>
</table>
Table V. Carbonyl levels. In sham and group I medulla spinalis carbonyl levels significantly higher than cortical and subcortical levels in the same group, p<0.05. Carbonyl levels significantly higher in all brain regions in group II than sham and group I, p<0.001

<table>
<thead>
<tr>
<th>Carbonyl levels</th>
<th>Cortical</th>
<th>Subcortical</th>
<th>Cerebellar</th>
<th>Medulla spinalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.57±0.13</td>
<td>0.50±0.03</td>
<td>0.69±0.01</td>
<td>1.22±0.03</td>
</tr>
<tr>
<td>Group I</td>
<td>0.70±0.01</td>
<td>0.73±0.02</td>
<td>0.84±0.02</td>
<td>1.25±0.03</td>
</tr>
<tr>
<td>Group II</td>
<td>1.22±0.03</td>
<td>1.22±0.03</td>
<td>1.21±0.03</td>
<td>1.69±0.01</td>
</tr>
</tbody>
</table>

group and group I (p<0.001). In sham group and group I, medulla spinalis levels were found to be significantly higher than cortical and subcortical levels in the same group. Increase in protein carbonyl levels in medulla spinalis tissue was much more prominent than cortical and subcortical levels in group II (Table V).

Results of ICP monitoring

In the sham group, the levels of ICP ranged between 4-8 mmHg. Group I had significant higher ICP values than sham group, they ranged between 10-12 mmHg (p<0.05). Group II had higher ICP values than sham group, they ranged between 11-13 mmHg (p<0.05).

Pathology of the brain tissue

There’s no intracranial hemorrhage at macroscopic evaluation of the brain tissue at sham group, but two in study group I (20%) and three in study group II (30%).

The mortality rate was 10% for sham and study group I and 20% for study group II.

Discussion

Abdominal compartment syndrome is defined as a marked increase (>20-25 mmHg) in IAP that occurs in some patients with multiple trauma, hemoperitoneum, severe forms of acute pancreatitis, post-abdominal surgery, or after massive colloidal or cristalloid fluid infusion. It has been demonstrated to affect multiple organ systems including the cardiovascular, respiratory, gastrointestinal, genitourinary and neurological systems [23-25]. Animal studies [10, 26, 28] have demonstrated that an acute increase in IAP can also cause a significant increase in ICP. Also, some clinical observation are thought that increase in ICP during abdominal trauma or surgery must also be considered a part of the ACS [2, 11, 14, 29]. It is difficult to monitor ICP in small animals such as rats; many kinds of ICP monitoring methods have been described. Barth et al. (1992) showed ICP monitoring from cisterna magna in rats, which is simple, reliable, and may be one of the best of the current ICP monitoring systems in the rats [30]. IAH causes elevation of diaphragm, increase of ITP, increase of pressure in great veins, and increase of ICP in accordance to Pascal's law [2, 5, 8, 31]. Decreased cardiac output (CO) due to IAH in addition to increased ICP causes more decrease in CPP [32]. These events cause secondary brain injury in patients who already have impaired cerebral autoregulation because of primary brain injury [32]. We must not forget that primary ischemic brain injury can cause dysphagia, gastrointestinal (GI) dysmotility and GI hemorrhage because of interruption of the axis between central nervous system (CNS) and GI system (GIS) [33]. This can show the close relationship and interaction of CNS and GIS.

Acute increase of IAP may be the cause of neurologic morbidity in trauma patients with or even without obvious signs of head trauma [2, 7, 12, 14]. Increase in IAP immediately causes a significant increase in ITP, in CVP and in ICP [11], and abdominal decompression resulted in normalization of ICP in both clinical [2, 12, 14] and experimental studies [10, 13]. This phenomenon becomes extremely important in light of the fact that, in a large prospective multicenter trial, 20% of patients with severe abdominal injuries and 40% of patients with severe intracranial injuries were documented as having an associated head or abdominal injury of similar magnitude, respectively, and that mortality in these patients was significantly higher than in patients without combined injury [34].

Although there are clinical and experimental studies about the effects of IAH and ACS on changes of hemodynamic parameters, ICP and CPP, there is no study about cerebral biochemical changes in literature. Oxidative stress procedure must be performed because of the decrease of CPP and increase of ICP due to a volume expansion by obstruction of venous outflow of the central nervous system. Therefore, we planned an experimental study evaluating oxidant-antioxidant status of various brain regions and medulla spinalis after IAH in two different pressures.

The normal values of IAP are subatmospheric to 0 mmHg [7]. The IAP level causing ACS is controversial. Recent animal data suggested that
Biochemical changes representing oxidative stress on brain tissue due to intraabdominal hypertension in a rat model

Prior shock and resuscitation may actually reduce the threshold levels of IAP that cause systemic manifestations of ACS [18, 35]. However, it is generally accepted that the patients with IAP in 20 mmHg must be closely followed, and that the ones with IAP in 25 to 30 mmHg require surgical decompression [2, 36]. Therefore, IAP levels in 20 and 30 mmHg were chosen in this study.

Total NO levels, XO activity, TBARS, protein carbonyls and protein SH levels were determined in various brain regions and spinal cord to show oxidative stress of central nervous system due to IAH in this study.

Although several sources for reactive oxygen species exist, attention has focused on XO and neutrophilic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [15]. They are enzymes producing superoxide. Superoxide own is a toxic oxidant metabolite besides it causes to produce hydrogen peroxide, another toxic metabolite [15]. Hydrogen peroxide may act as a fuel for myeloperoxidase-catalyzed reactions. MDA, which arises from the breakdown of lipid peroxyl radicals, is one of the indicators of oxidative stress [15]. MDA is also important in that it can cause further oxidative injury by oxidizing protein molecules [37, 38]. Another indicator of oxidative stress is the carbonyl content of proteins, resulting from the oxidation of specific amino acid residues [39]. On the other hand, glutathione systems, such as intracellular glutathione sulfhydryl (GSH) or sulfhydryl (SH) groups of proteins in extravascular areas, are considered to represent one of the major mechanisms of reducing oxidative stress. Thus increased NO, XO, MDA and protein carbonyl levels and loss or oxidation of GSH provide physiologically relevant estimates of oxidative stress in tissues.

In the study, TBARS levels, an indicator of MDA, NO levels, XO levels, and carbonyl levels of the various central nervous system regions were increased, and SH levels were decreased after IAH. These results supported the observation that IAH also affects the central nervous system as pulmonary, cardiac, renal and hepatobiliary systems, and IAP causes detrimental effects on biochemical parameters. The biochemical results of our study showed that the biochemical findings of oxidative stress due to IAH in various regions of the central nervous system appeared after IAH for only one hour. In other words, acute increase of IAP may cause complications in the central nervous system and secondary neuronal injury in a short time (60 minutes). These findings are convergent with the results of the study of Citerio et al. [11]. This study shows that an increase in IAP immediately causes an increase in ITP, CVP and ICP. Therefore, aggressive treatment of IAH is important to prevent such complications in the patients with concomitant abdominal and head injuries.

Another interesting finding in this study was that higher IAP generally caused more detrimental effects on the parameters evaluated. These results suggest that higher IAP causes more detrimental effects on the central nervous system.

The levels of spinal cord and cerebellum samples were significantly worse in the study groups for most of the markers. For TBARS, NO, carbonyl, the levels of spinal cord in group II, and for XO, the levels of cerebellum and spinal cord in groups I and II were found to be higher than those of levels of other regions in the same group. These results suggested that spinal cord might be affected by compression of lumbar vertebral venous pressure due to IAP in addition to increase of ICP. The study of Josephs et al. [26] supported these findings.

The levels of ICP of study groups were significantly higher than sham group. These results suggested that intracranial space affected by the compression of great veins pressure due to increased IAP.

The study has some limitations. This is an initial study for ACS that affects ICP. However, for the reason that many other parameters should be still monitored, such as blood gases, venous blood return from brain, the study should be treated as a preliminary report. We plan to continue the research in our next studies.

Conclusions

IAH and ACS may cause biochemical changes representing oxidative stress on various regions of the central nervous system. Even 60 minutes after an increase of IAP and a higher IAH caused more detrimental effects. Most prominent effects were seen in the spinal cord and cerebellar tissue suggesting that compression of lumbar vertebral venous pressure may have a role in addition to increase of ICP due to increase of pressure of great veins caused by increase of ITP depending IAH. Because of these surgeons and intensive care physicians must be care about ACS and secondary ICP increasing.

References