# Restraint Stress Aggravates Intestinal Mucosal Barrier Damage in Rats after Cerebral Stroke

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**Abstract: Background** Complications such as gastrointestinal disorders may occur in patients after stroke, which affect their recovery. It is still obscure whether the stress from movement inabilities after stroke influences the intestinal barrier functions. We therefore assessed the intestinal mucosal changes in a post-stroke rat model under the restraint stress, which simulated movement restriction in post-stroke patients. **Methods** Male SD rats in stroke group (M) and post-stroke restraint group (MS) received permanent middle cerebral artery occlusion (MCAO) operation to establish a stroke rat model, rats in post-stroke restraint group (MS) were restrained in fixator 24 hours later after MCAO operation. The rats in sham-operated restraint group (JS) received restraint only after sham operation. Serum corticotrophin releasing factor(CRF), D-lactate (D-Lac), endotoxin (LPS) and diamine oxidase (DAO) level were evaluated by ELISA assay; Terminal ileum tissue tight junction proteins ZO-1, occludin, claudin-1, claudin-2, claudin-3 and claudin-4 level were analyzed by western blotting; Terminal ileum tissue CRF, CRF-r1, CRF-r2 and brain tissue TNF-α, CRP, CRF expression level were investigated by immunohistochemistry. **Results** As compared with sham group (J), peripheral blood serum D - Lac, DAO, LPS, CRF levels increased (all $P<0.05$), TNF-α, CRP, CRF expression levels in the central nervous system were also significantly increased ($P<0.01$), while the claudin, ZO-1, claudin-1, claudin-2 levels in the intestinal mucosa decreased significantly (all $P<0.01$) in post-stroke restraint group (MS), stroke group(M) and sham-operated restraint group (JS). Peripheral blood serum DAO, LPS, CRF levels in post-stroke restraint group (MS) were increased compared with stroke group (M) (all $P<0.05$). The ZO-1, claudin-1, claudin-2 levels in intestinal mucosa were decreased compared with stroke group (M) (all $P<0.01$), while claudin-3 claudin-4 levels were increased compared with stroke group (M) (all $P<0.01$). Terminal ileum tissue CRF-r1 expression levels in post-stroke restraint group (MS) was increased compared with stroke group (M) ($P<0.05$), while CRF-r2 expression level was no significant difference in this two groups ($P>0.05$). **Conclusion** Restraint stress deteriorates the intestinal barrier functions in rats after stroke.
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**Keywords:** restraint stress; intestinal permeability; intestinal mucosal barrier; HPA axis

## 1. Introduction

Stroke is a common disease in clinical work. The post-stroke patients often have varying degrees of movement disorders, in bed and activity limitation status, supervened with pressure sores, infections, gastrointestinal disorders and other complications, which affect the recovery. Whether this stress from movement inabilities after stroke influences the intestinal barrier functions needs further investigation.

Study has confirmed chronic restraint stress can affect the local cerebral ischemic rats' motor function recovery, either given restraint before ischemic or after [1]. Chronic stress is related to increased stroke risk and increases stroke vulnerability by impairing endothelial function [2].

Corticotropin releasing hormone (CRH) as a peptide hormone and neurotransmitter, involved in the stress response, potentiate excitotoxic damage in the brain and modulate inflammatory responses in the periphery through two known CRF receptors, CRF-r1 and CRF-r2 [3]. Cerebral ischemia impaired the local intestinal immune populations which are a part of gut barrier function, cause a reduction of T and B cells in the Peyer's patches [4]. Other studies figured out that CRH exacerbated the injury via CRH-r1 after stroke, though the mechanism keeps unclear [3]. CRF peptidergic system modulated cerebral blood flow in ischaemic stroke [5], while the role of CRF and its receptors in the intestinal barrier functions after stroke still keeps unknown.

In this study, we established a stoke rat model with restraint stress to simulate activity limitation stroke patients in clinic [6], intestinal mucosa tight junction proteins ZO-1, occludin, claudin-1, claudin-2, claudin-3 and claudin-4 expression and periphery CRF, CRF-r1, CRF-r2 in the intestine and brain tissue TNF-α, CRP, CRF expression level were determined. The results in this study indicated that restraint stress might deteriorate the intestinal barrier functions through CRH-r1 in rats after stroke.

## 2. Materials and Methods:

### Rats:

Male Sprague-Dawley rats were purchased from the Beijing Vital River Experimental Animal Technology Co (Beijing, China, animals Certificate Number: 11400700023659), weighing 200 ~ 220g. Adaptive feeding one week before the experiment, each cage 3. Restraint group were single solitary cage support, sham and stroke group 3 per cage feeding. Before the experiment began with Open-Field Behavior scoring method[7] to filter the rats, the similar score data rats were randomly divided into four groups: sham group (J), sham-operated restraint group (JS), stroke group (M), post-stroke restraint group (MS), n = 12.

### Development of a rat model with ischemic stroke:

Ischemic stroke rat model was established by modified Longa suture method [8]. Firstly, fasted and baned water for 12 hours preoperative, anesthetized by intraperitoneal injection 1.25% tribromoethanol (concentration of 0.2ml/10g weight). The left common carotid artery (CCA) was exposed through a midline incision. The external carotid artery(ECA) and internal carotid artery(ICA) were isolated and separated from the neck muscles. The CCA was ligated with 5-0 silk suture close to its origin. The terminal branches of the CCA was placed a slipknot close to the bifurcation between ICA and ECA. A 5-0 silk suture around the CCA was placed between the two ligature. Secondly, the CCA was cut a small incision with microvascular scissors at the position between the slipknot and the silk suture around the CCA. A 7-cm length of fishing line (D = 0.285mm), its tip rounded by dipped with wax, was introduced into the CCA lumen through the incision. The silk suture around the CCA stump was tightened around the fishing line to prevent bleeding. Slightly loosened the slipknot, the fishing line was then gently advanced from the CCA to the ICA lumen. After a variable length of fishing line had been inserted into the CCA stump, resistance was felt, indicating that the blunted tip of the fishing line had passed the middle cerebral artery(MCA) origin. At this point, the intraluminal fishing line has blocked the origin of the MCA. About 1cm fishing line exposure to the CCA was kept, the rest was cut off. The incision was closed. Neurobiology behavior changes were observed after rats awake. The neurobehavioral evaluation criteria was that[8]: a score of 0 indicated no neurologic deficit, a score of 1 (failure to extend right forepaw fully) a mild focal neurologic deficit, a score of 2 (circling to the right) a moderate focal neurologic deficit, and a score of 3 (falling to the right) a severe focal deficit; rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness. The rats scored 1~3 points were considered effective ischemic stroke models.

### Restraint stress rat model:

24 h after operation, rats' neural function basically recovered, vital signs stabled, rats were placed into rat fixators, 2 hours per day, 7 days in a row, the daily restraint time was not fixed, at 08:00 ~ 18:00. Fasting but watered during the process.

### Assessment of intestinal barrier function:

At the end of test, collected 1 ml blood from rats' orbital venous plexus, kept serum after centrifugation, inspected the serum Corticotrophin releasing factor(CRF), D-lactic acid (Lac), endotoxin (LPS), diamine oxidase (DAO) levels by ELISA. Rapid anesthetized rats after collecting blood (anesthesia method with former), taken 3-4 cm terminal ileum tissue (2 cm from the ileocecal junction) of rats, sheared 0.2 cm segments into protein cracking liquid, extracted intestinal tissue protein after grinding with a grinding rod, measured the tight junction proteinZO-1, blocking protein Occludin-1, blocking protein claudin 1, claudin - 2, claudin - 3, claudin - 4 levels of the terminal ileum by western blot method.

### Immunohistochemical method:

Rapid anesthetized rats(as aforesaid),were taken 3-4 cm terminal ileum tissue (2 cm from the ileocecal junction), cut skull open to take out full brain tissue, terminal ileum tissue and brain tissue were clearned in PBS buffer, immersed in 4% paraformaldehyde, conventional dehydrated, paraffin embedded, sectioned, dyed by immunohistochemical method (SP), CRF, CRF - r1, CRF - r2 expression level in terminal ileum tissue, TNF-α, CRP expression level in the hippocampus tissue, CRF expression level in the hypothalamus area tissue were analyzed.

### Statistical analysis:

The data was expressed as mean ± standard deviation (SD). All analyses were performed using SPSS l 9.0 software for windows. Comparison between groups was analyzed by one-way analysis of variance or *t* test. *P* values of less than 0.05 were considered statistically significant.

## 3. RESULTS:

### CRF, D - Lac, LPS, DAO level in the peripheral blood serum:

In Comparison with the sham group (J), DAO levels in MS group, M group were significantly increased ($P < 0.01$), while DAO level in JS group was no significant difference ($P>0.05$); D–Lac, LPS and

CRF levels in MS group, M group and JS group were significantly increased (all $P < 0.01$). The DAO, LPS and CRF levels in MS group were increased compared with M group (all $P < 0.05$), while the D–Lac Level was no significant difference between MS group and M group ($P>0.05$) (shown in table 1). These results showed that the intestinal mucosa permeability increased intestinal function changes in rats with stroke after restraint stress.

**The tight junction protein expression levels in the terminal ileum:**

In Comparative with the sham group (J), tight junction protein ZO-1, claudin-1, claudin-2 levels in MS group, M group and JS group were significantly decreased (all $P < 0.05$); tight junction protein Occludin level in MS group was significantly decreased ($P < 0.01$), while Occludin expression between M group, JS group were no significant difference (all $P>0.05$); claudin-3 levels in MS group, M group, JS group were significantly increased(all $P< 0.05$); claudin-4 levels in MS group, M group increased greatly (all $P<0.01$), while claudin-4 in JS group had no significant difference($P>0.05$). The tight junction protein ZO-1, claudin-1, claudin-2 levels of MS group were decreased compared with M group(all $P<0.05$), the claudin-3, claudin-4 levels were significantly increased compared with M group(all $P<0.01$).The results suggested that, restraint stress after stroke could decrease ZO-1, claudin-1, claudin-2 expression levels (shown in table 2, figure 1).

Table 1: Serum CRF, D-Lac, LPS, DAO level(*Mean*±*SD*)

| | M | MS | JS | J |
|---|---|---|---|---|
| CRF(ng/ml) | 170.6±18.09* | 194.0±19.8 | 189.0±7.45 | 135.4±6.57 |
| D-Lac(μg/ml) | 1231±109.0 | 1292±133.6 | 1114±108.7b | 978.0±95.33 |
| LPS(μg/ml) | 139.6±5.04* | 147.0±3.16 | 132.7±7.34b | 117.0±7.93 |
| DAO(pg/ml) | 612.7±40.93* | 694±55.38 | 563.2±32.5b | 545.0±17.69 |

*$P<0.05$, **$P<0.01$ for MS group vs M group; a $P<0.05$, b $P<0.01$ for MS group vs JS group.

Table 2: The terminal ileum tissue ZO - 1, Occludin, claudin protein level (*Mean*±*SD*)

| | M | MS | JS | J |
|---|---|---|---|---|
| ZO-1 | 0.64±0.07* | 0.52±0.05 | 0.74±0.08b | 0.86±0.06 |
| Occludin | 0.67±0.06* | 0.58±0.03 | 0.65±0.05b | 0.71±0.06 |
| claudin-1 | 0.71±0.07** | 0.57±0.05 | 0.70±0.09a | 0.80±0.05 |
| claudin-2 | 0.58±0.08** | 0.35±0.07 | 0.54±0.07b | 0.71±0.06 |
| claudin-3 | 0.48±0.05** | 0.59±0.04 | 0.44±0.04b | 0.38±0.04 |
| claudin-4 | 0.46±0.04** | 0.58±0.03 | 0.36±0.03b | 0.31±0.07 |

*$P<0.05$, **$P<0.01$ for MS group vs M group; a $P<0.05$, b $P<0.01$ for MS group vs JS group. Variations of protein levels = target protein /β-actin.

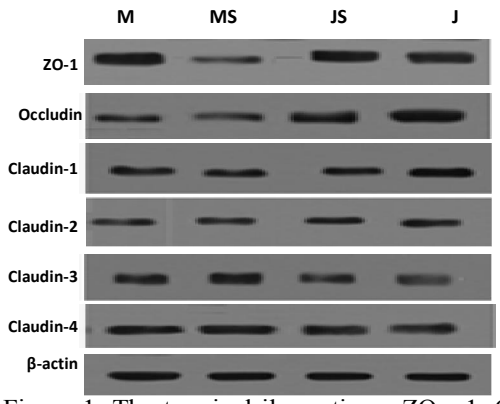


Figure 1: The terminal ileum tissue ZO - 1, Occludin, claudin protein level

**CRF, CRF - r1, CRF - r2 expression in terminal ileum tissue:**

In Comparative with the sham group (J), CRF, CRF-r1, CRF-r2 expression levels in MS group, M group and JS group were significantly increased (all $P<0.05$). CRF-r1 expression level in MS group was significantly increased compared with M group($P< 0.01$); while CRF and CRF- r2 expression levels were no significant difference in this two groups ($t$ =1.584, 1.194, all $P>0.05$). CRF - r1 / CRF - r2 in each group had no significant difference ($F$= 1.616, $P > 0.05$) (shown in table 3 and figure 2). The results showed that local increased CRF-r1played an important role in intestinal permeability changes caused by restraint stress after stroke.

Table 3: CRF, CRF - r1, CRF - r2 positive cells expression in terminal ileum tissue (*Mean±SD*)

| | M | MS | JS | J |
|---|---|---|---|---|
| CRF | 147.3±20.54 | 173.8±36.42 | 87.50±17.64[b] | 63.47±11.84 |
| CRF-R1 | 123.6±24.06[**] | 169.6±26.99 | 101.7±19.79[b] | 66.06±11.54 |
| CRF-R2 | 125.4±24.35 | 141.2±34.05 | 88.39±19.38[b] | 70.73±17.89 |

*$P<0.05$, **$P<0.01$ for MS group vs M group; a $P<0.05$, b $P<0.01$ for MS group vs JS group. Positive cells expression values were shown in mean optical density value (Density), analyzed grey values by Spearman rank correlation.

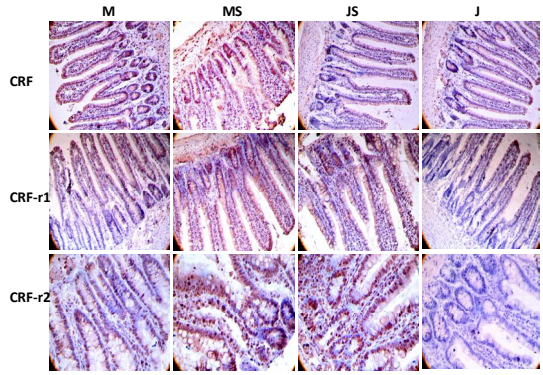


Figure 2: CRF, CRF - r1, CRF - r2 positive cells (immunostaining in brown) expression in terminal ileum tissue

**TNF-α，CRP，CRF positive cells expression in central nervous system:**

In Comparative with the sham group (J), TNF-α, CRP, CRF expression levels in MS group, M group and JS group were significantly decreased（$P<0.01$). CRF expression level in MS group was significantly increased compared with M group($P<0.01$); while TNF-α and CRP expression levels were no significant difference compared with M group (all $P>0.05$) The results indicated that restraint after stroke affected the regulation of CRF secretion by central nervous system, but it couldn't increase the expression levels of TNF-α and CRP in brain (shown in table 4 and figure 3).

Table 4: TNF-α，CRP，CRF positive cells expression in central nervous system (*Mean±SD*)

| | M | MS | JS | J |
|---|---|---|---|---|
| TNF-α | 140.3±20.56 | 148.3±31.88 | 62.20±10.56[b] | 50.88±6.163 |
| CRP | 106.9±25.67 | 135.3±40.67 | 82.42±15.37[b] | 57.19±5.594 |
| CRF | 103.1±20.98[**] | 115.3±17.79 | 80.03±17.14[b] | 51.46±3.663 |

*$P<0.05$, **$P<0.01$ for MS group vs M group; a $P<0.05$, b $P<0.01$ for MS group vs JS group. Positive cells expression values were shown in mean optical density value (Density), analyzed grey values by Spearman rank correlation.

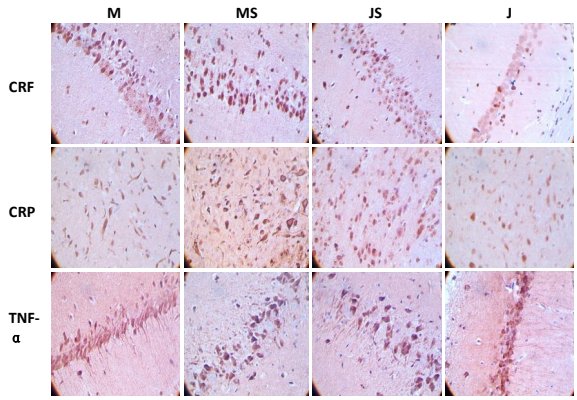


Figure 3: CRF, CRP, TNF-α positive cells (immunostaining in brown) expression in central nervous system

## 4. DISCUSSION

This study has the following major findings: (1) the restraint stress after ischemic stroke induced a marked increase in LPS and DAO levels of peripheral blood serum, which reflected the increase of intestinal mucosal permeability; (2) the restraint stress after ischemic stroke induced the tight junction protein ZO-1, claudin-1, claudin-2 levels of the terminal ileum decreased, which reflected the destruction of gut barrier integrity; (3) the change trend in claudin-3, claudin-4 level was opposite to claudin-1, claudin-2 level, suggested that the tight junction protein claudin-3 and claudin-4 may be associated with the damage of intestinal mucosa barrier; (4) the restraint stress after ischemic stroke induced a marked increase in peripheral blood serum and central nervous system CRF expression levels, the CRF-r1 expression level in local intestinal was significant increase at the same time, suggested that restraint stress after ischemic stroke may lead to "HPA axis" abnormally activated,

the central nervous system CRF secretion increase, CRF-r1 level in local intestinal was up regulated, peripheral blood CRF level elevated ultimately. The abnormally increase of CRF level may be one of the mechanism of intestinal mucosal barrier damage in post-stroke rat under the restraint stress.

The previous experimental research confirmed ischemic stroke induced intestinal mucosa barrier change, tight junction protein of Occludin and ZO - 1 expression decrease, accompany with the tight junction damage. Destruction of tight junction maybe one of the molecular mechanisms of pathogenesis of intestinal harrier dysfunction in ischemic stroke[9] . Intestinal mucosal permeability is an important symbol of intestinal mucosal integrity and gut barrier function, the permeability change of the intestinal helps to find the early damage of intestinal mucosal barrier [10]. D-lactic acid (D-Lac) and diamine oxidase (DAO) are often used in the detection of intestinal mucosal permeability index. The activity of DAO and concentration of D-Lac in serum can reflect the degree of intestinal barrier damage timely and effectively in the condition of ischemic stroke, which could be acting as early-warning index of intestinal mucosal damage [11, 12]. Unusually high concentration LPS in serum show the existence of bacteria and endotoxin translocation after intestinal barrier damaged, this could indirectly reflect the degree of intestinal barrier damage[13].

CRF positive neurons are widely distributed in the rat brain，the mRNA are mainly distributed in the hypothalamus paraventricular nucleus, the cerebral cortex, hippocampus and amygdala nucleus, etc[14].CRF system have expressed throughout the gastrointestinal tract. They play a role in intestinal mucosal damage and relate to intestinal micro-ecosystem balance[15]. Stroke related intestinal gut barrier damage ; was positive correlation with CRF protein level in hypothalamus and intestinal tissue[16] has been confirmed. Other studies figured out that CRF exacerbated the injury via CRF-r1 after stroke. It suggested that gastrointestinal barrier damage in stroke might be associated with stress.

Based on the previous studies, we established the ischemic stoke rat model with restraint stress, to research the post-stroke rats' intestinal mucosal barrier change under the restraint stress. The peripheral blood serum D - Lac, LPS and DAO levels were selected to reflect the change of intestinal mucosal permeability; the terminal ileum tight junction protein ZO-1, caudin-1and claudin-2 levels to reflect the intestinal harrier dysfunction. Because of previous research absence, the tight junction protein caudin-3 and claudin-4 were selected at the same time.

Our study results showed that, the degree of intestinal barrier damage was aggravated in post-stroke restraint group (MS) rat further, shown up as the permeability increase represented by the increase of serum LPS and DAO levels. The results prompted that stroke and movement restricted patients existed intestinal mucosa barrier damage and intestinal permeability increase, which leading large quantities of LPS entered blood circulation. The D-Lac level in peripheral blood serum in our study was no significant difference between MS group and M group, the result didn't agree with our expectation. Preliminary analysis suggests that the restraint induced rats movement restricted, reduce the anaerobic metabolism of rats, it might partly offset D-Lac increase which caused by intestinal mucosal permeability increase, and prevent further rise of D-Lac level. It still needed further study.

The change of tight junction proteins claudin-1, claudin-2 level was consistent with serum LPS、DAO level and terminal ileum tissue tight junction proteins ZO-1 and ocludin level, this suggested that tight junction proteins claudin-1, claudin-2 play a protective role in intestinal mucosal barrier function. while the change trend in claudin-3、claudin-4 level was opposite to claudin-1、claudin-2 level, suggested they may be associated with the damage of intestinal mucosa barrier. There was evidence that claudin-4 was relate to the endothelial junctions of intestinal epithelium expanded and membrane channel opened, over-expressed claudin–4 increased permeability of the intestinal mucosa [17]. The conclusion showed a good agreement with our experimental results, prompt over-expression of claudin-3 may cause intestinal mucous permeability increased permeability of the intestinal mucosa, increase the risk of intestinal mucosa injury and endotoxemia. The mechanism is needed further research.

CRF expression in the hypothalamus, terminal ileum and peripheral blood were determined in our study to reflect the various levels of hypothalamus pituitary adrenal (HPA) axis' secretion changes. The results showed that the restraint stress after ischemic stroke induced a marked increase in peripheral blood serum and central nervous system CRF expression levels, while the CRF-r1 expression level in local intestinal was significant increase. This may be the endocrine mechanism of the restraint stress after ischemic stroke related intestinal gut barrier damage. It suggested that the stroke and movement restricted patients may exist CRF secretion and intestinal sensitivity further rise compared with the stroke patients without movement restricted. The expression levels of TNF-α and CRP in brain were no significant difference in MS group and M group, suggested that the restraint stress couldn't change the

central nervous system TNF-α and CRP expression levels in post-stroke rats.

In conclusion, restraint stress and stoke could lead to intestinal epithelium cellular tight junction (TJ) damage and the integrity of intestinal mucosa barrier breaking in rats. Restraint stress after stroke aggravated the intestinal mucosal barrier damage. The mechanism might be related to "HPA axis" abnormal activation, increased central and peripheral CRF, and enhanced intestinal CRF-r1 expression.

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