

# **ACTIVATION OF THE HYPOTHALAMIC– PITUITARY–ADRENAL AXIS INDUCES THE DIFFERENTIAL RELEASE OF PRO– INFLAMMATORY CYTOKINES IN BALB/C MICE**

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## **Abstract**

Physiological processes are associated with interactions between the nervous, endocrine and immune systems, which communicate through neurotransmitters, hormones and cytokines. The key to this communication is the hypothalamic–pituitary–adrenal (HPA) axis which can be activated by stress and pro–inflammatory cytokines. We have investigated the effects of acute and chronic stress on the release of pro–inflammatory cytokines through activation of the HPA axis. Accordingly, BALB/c mice were exposed to acute stress through the single application of bi–frontal electrical stimulation (ESa, 20V/10 mA/0.05s) and the effect produced by a single i.p. dose of Lipopolysaccharide (LPS) (250 µg/100g). Chronic stress was (ESc) induced by applying the electrical stimulus to the animals in the same way for 7 consecutive days, as well as the effects of ascending LPS administration over 7 days (35–250 µg/100g i.p./day). Following stimulation, the levels of pro–inflammatory cytokines and corticosterone were quantified by ELISA. Unlike LPS that provoked an increase in TNF– $\alpha$ , IL–1 $\beta$ , IL–6, and corticosterone levels, acute electrical stress did not induce any change in the levels of pro–inflammatory cytokines or corticosterone. In contrast, chronic ESc stress produced an increase in the levels of TNF– $\alpha$ , while in the animals treated with LPS the IL–6 levels increased. These data suggest that the response of the HPA axis to stress depends on the duration, intensity and etiology of the stress agent, also suggest a suitable model for the evaluation of drugs with potential effect on inflammatory stress.

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**Keywords:** HPA axis, pro-inflammatory cytokines, LPS, electrical stimulation, corticosterone

## **Introduction**

The homeostasis in organisms is maintained through a complex dynamic equilibrium that is constantly challenged by adverse intrinsic or extrinsic forces, stress factors (Tsigos & Chrousos, 2002). Stress can be defined as a constellation of events that involve a stimulus (stressor), which precipitates a reaction in the brain (stress perception) and that subsequently activates physiologic fight systems in the body (stress response) (Dhabhar, 2002), thereby provoking a state of threatened homeostasis (Chrousos, 1995; Peterson, Chan, & Molitor, 1991). Stress activates the HPA axis (Black, 2002) and in response to physiological and stressful stimuli, this axis stimulates the release of the corticotrophin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus, provoking the synthesis of adrenocorticotrophic hormone (ACTH). In the adrenal gland this hormone stimulates the released of glucocorticoids (GC), typically cortisol in humans and corticosterone in rodents (Engelsma et al., 2002). It has also been suggested that both a stress stimulus and activation of the immune system leads to the stimulation of common neuronal pathways (A. J. Dunn, Wang, & Ando, 1999; A. J. Dunn & Welch, 1991). Accordingly, acute stress activates the sympathetic nervous system as well as the HPA axis (Connor, Kelly, & Leonard, 1997; McCarty, Kvetnansky, & Kopin, 1981), and the final products (catecholamines and glucocorticoids) are often implicated as mediators of stress-induced immunosuppression (Connor, Brewer, Kelly, & Harkin, 2005). Activation of the innate or non-specific immune system, results in the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 (IL-1) and interleukin-6 (IL-6) by phagocytic cells. During infection these pro-inflammatory cytokines not only stimulate inflammation of the infected site but they also signal to the brain, provoking the activation of regions involved in neural mediated components of the hosts defense (A. J. Dunn, 1993; Johnson, O'Connor et al., 2002). These cytokines, either alone or in conjunction with components of the stress system and the classic stress hormones, induce fever, sleepiness, fatigue, loss of appetite and decreased libido, and they activate the hepatic synthesis of acute phase proteins. In conjunction, these changes are referred to as "sickness behavior" and "acute-phase response", respectively.

The stress that is associated with an immune challenge has been called immune or inflammatory stress, and like other forms of stress it is coordinated by the central stress system and its peripheral arm (Chrousos, 1995). In a previous study, we reported that the induction of stress in the rat

by electrical shock over seven days increased the corticosterone levels associated with the suppression of cellular immune responses (Villasenor-Garcia, Puebla-Perez, Sandoval-Ramirez, & Lozoya, 2000). In another study, electrical stimulation of the afferent vagus nerve induced brain IL-1 $\beta$  expression, but no IL-1 $\beta$  was detected in the plasma. Likewise, this stimulation regime increased the corticosterone levels and activated the HPA axis (Hosoi, Okuma, & Nomura, 2000). In the present study, we investigated the effect of physical and immune stress on pro-inflammatory cytokine release through activation of the HPA axis.

## **Materials and Methods**

### *Animals*

Male BALB/c mice (eight week old, 20–25 g) were selected and housed under standard conditions at ambient temperatures of 22–24 C, a 12:12 h light–dark cycle, and with ad libitum access to food and water. At all times, animals (n=10 per cage) were manipulated according to the guidelines on the use and care of experimental animals (Mexican Official Norm NOM–062–ZOO–1999 published by SAGARPA in the *Diario Oficial del Gobierno Mexicano* paper on June 28, 2001).

### *HPA axis activation*

#### *Stress by electrical stimulation*

Animals were exposed to an electrical stimulus 20 V, 10 mA, and 0.05 s applied through the skin of the frontal areas of the head. Electrical stimulation was considered as acute physical stress when delivered once, and animals exposed to seven consecutive sessions were considered as having been subject to chronic physical stress (Villaseñor-Garcia, Lozoya, & Puebla-Perez, 2001; Viveros-Paredes, Puebla-Pérez, Gutiérrez-Coronado, Sandoval-Ramírez, & Villaseñor-García, 2006).

#### *Stress by LPS administration*

To induce immune stress, LPS (*Escherichia coli*, 055:B5; sigma) was dissolved in sterile endotoxin-free PBS vehicle was injected intraperitoneally (i.p.). When acute stress was induced, a single a dose of 250  $\mu$ g/kg was administered, while chronic LPS administration involved ascending doses of 35, 70, 105, 140, 175, 210 and 250  $\mu$ g/100g ip/day over 7 days (Akmaev & Grinevich, 2001)

### *Controls*

The Control groups (Ct) in this study were comprised of healthy mice that were not exposed to stress.

All animals were treated once daily, between 9:00 and 10:00 h, for one or seven consecutive sessions. One hour after the last session, the animals were mildly anesthetized with ether and sacrificed. The animals were treated in compliance with the indications of the Mexican NOM–062–ZOO–1999 routinely used in our laboratory

#### *Plasma collection*

Blood samples were taken 90 min after the last stress stimulus or administration of LPS and the whole blood was separated by centrifugation. Plasma was collected and stored at –80°C until the time of the assay.

#### *Measurement of TNF- $\alpha$ , IL-1 $\beta$ and IL-6 concentrations in plasma*

The concentrations of cytokines in plasma were measured using murine specific commercially available enzyme-linked immunosorbent assays (ELISA; R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The absorbance (OD<sub>450</sub>) was measured in a microtiter plate reader (BioRad). Cytokine concentrations (pg/mL) were calculated by extrapolating from standard curves.

#### *Corticosterone measurements*

The plasma corticosterone concentration was measured using a commercially available ELISA kit for mice following the manufacturer's instructions (IDS, London, England). The absorbance (OD<sub>450</sub>) was measured in an ELISA microtiter plate reader (BioRad). The corticosterone concentration (ng/mL) was determined by extrapolation from a standard curve prepared with known concentrations of corticosterone ( $r = 1.0000$ ).

#### *Statistical analysis*

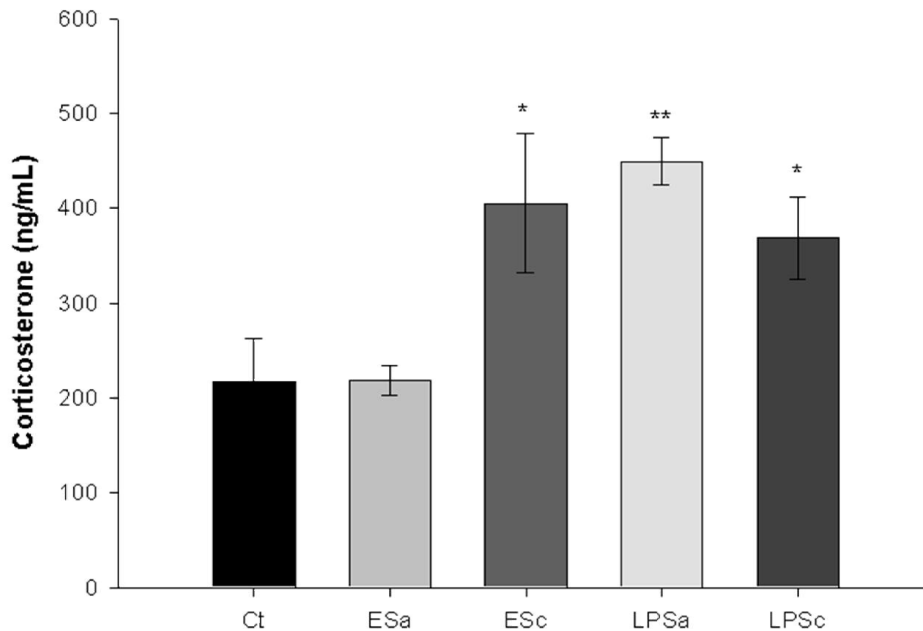
Values are expressed as the mean  $\pm$  S.D. The differences between groups were analyzed with ANOVA applying the T3 Dunnett's modification. A value of  $P < 0.05$  was considered to indicate statistical significance.

## **Results**

#### *Effect of stress on corticosterone levels*

The plasma corticosterone values of the different groups of mice only varied following the application of chronic electrical stress (fig. 1). In control animals the corticosterone levels reached  $217.87 \pm 44.70$  ng/mL and these levels were not altered in the group that suffered acute electrical stress. In contrast, serum corticosterone levels increased significantly when the electrical stress was chronic, reaching values of  $404.99 \pm 73.23$  ng/mL ( $P < 0.01$ ). However, exposure to a single acute and increasing chronic administration of LPS both produced a marked increase in corticosterone

levels ( $P<0.001$  and  $P<0.01$  respectively) when compared to the control animals.



**Fig.1.** Circulating corticosterone levels 90 min after acute (a) and chronic (c) ES application or LPS administration. Bars represent the mean  $\pm$  S.D. of the values obtained from 5 mice per group. \* $P<0.01$  and \*\* $P<0.001$  vs control.

#### *Effect of stress on the levels of proinflammatory cytokines TNF- $\alpha$*

TNF- $\alpha$  levels were significantly increased ( $P<0.0001$ ) by a single administration of LPS. In contrast, LPS administration in ascending doses over seven days did not alter the levels of this cytokine. On the other hand, the group exposed to acute electrical stress did not display any alteration in their levels of TNF- $\alpha$ , although chronic electrical stress provoked a significant increase in the levels of TNF- $\alpha$  in animals when compared to the control group ( $P<0.05$ , Fig. 2).

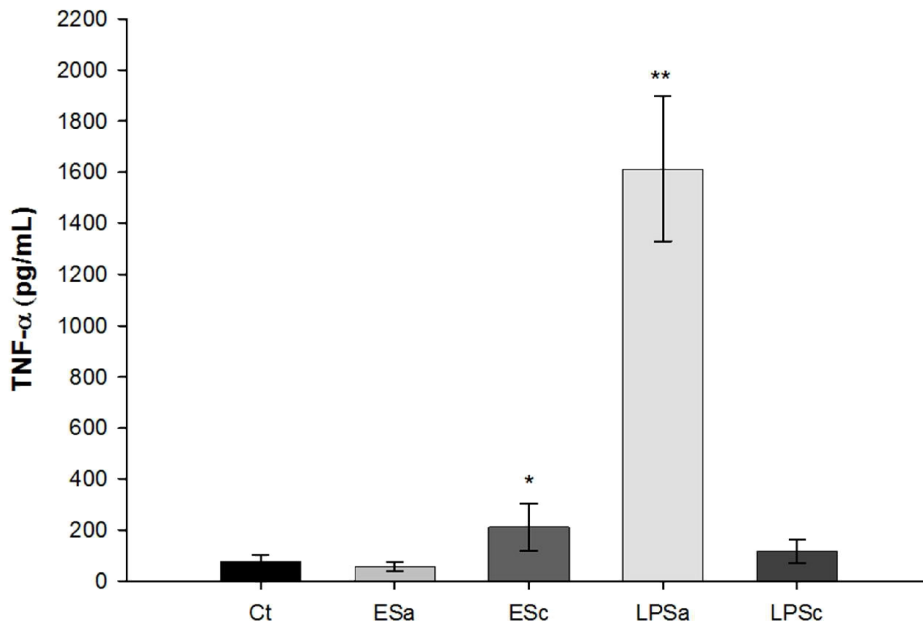


Fig. 2. Circulating levels of TNF- $\alpha$  90 min after acute (a) and chronic (c) ES application or LPS administration. Bars represent the mean  $\pm$  S.D. of the values obtained from 5 mice per group. \* $P$ <0.01 and \*\* $P$ <0.001 vs control.

#### *IL-1 $\beta$*

IL-1 $\beta$  levels were increased significantly after a single administration of LPS ( $P$ <0.05) although chronic LPS administration in ascending doses over seven days did not alter the levels of this cytokine when compared to the control animals. Neither acute nor chronic electrical stress altered the levels of this cytokine (Fig. 3).

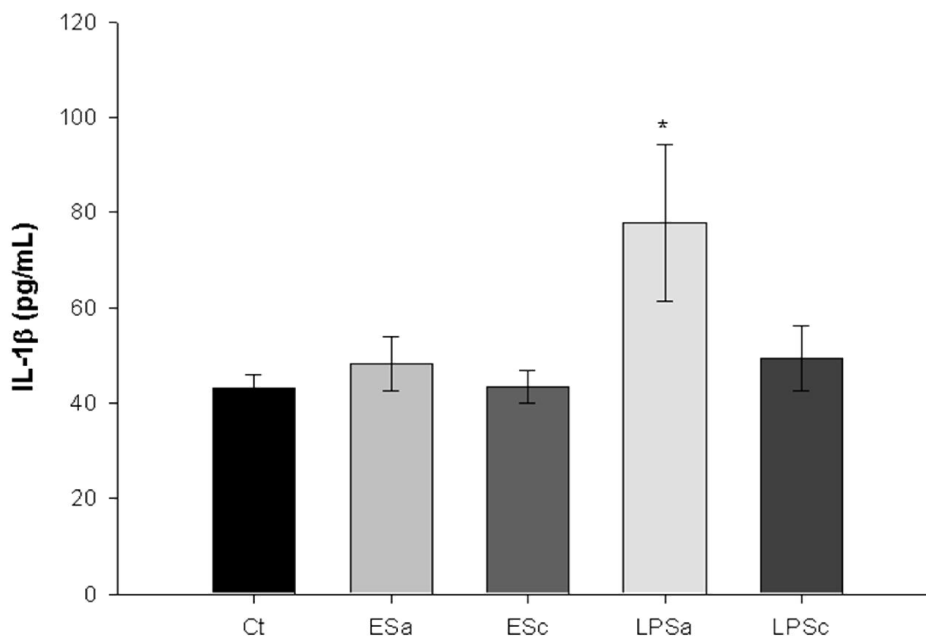


Fig. 3. Circulating IL-1 $\beta$  levels 90 min after acute (a) and chronic (c) ES application or LPS administration. Bars represent the mean  $\pm$  S.D. of the values obtained from 5 mice per group. \* $P$ <0.05 vs control.

### *IL-6*

The levels of IL-6 were significantly increased by a single acute injection of LPS ( $P$ <0.001). Similarly, chronic LPS administration in ascending doses over seven days provoked a significant increase in the levels of this cytokine when compared with the control group ( $P$ <0.05). On the other hand, the animals exposed to acute or chronic electrical stress did not display any alteration in the levels of this cytokine (Fig. 4).

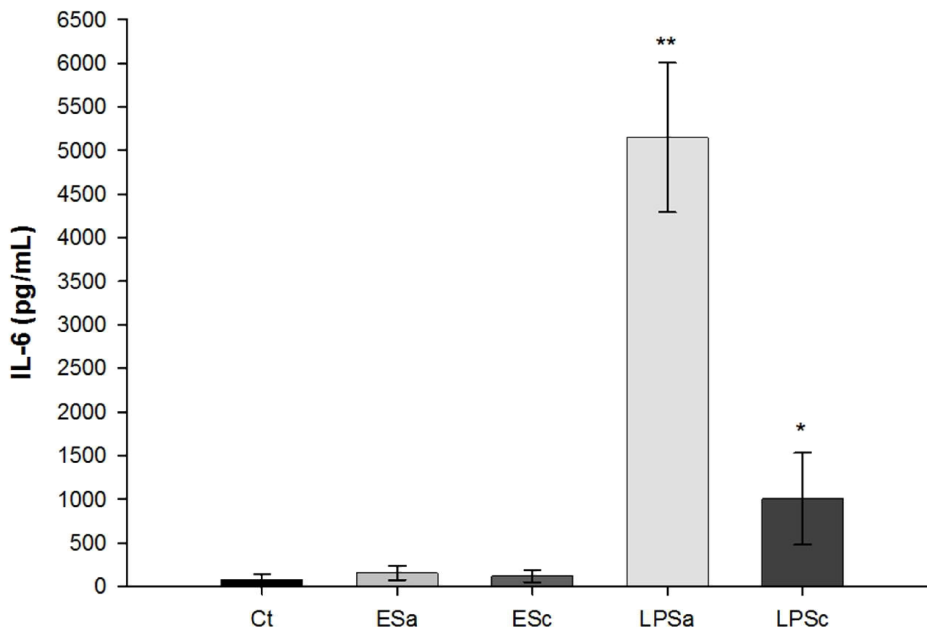


Fig. 4. Circulating IL-6 levels 90 min after acute (a) and chronic (c) ES application or LPS administration. Bars represent the mean  $\pm$  S.D. of the values obtained from 5 mice per group. \* $P$ <0.05 and \*\* $P$ <0.001 vs control.

## Discussion

Activation of the HPA axis is associated with increased levels of corticosterone, immunosuppression and disruption of the typical Th1/Th2 cytokine profile (Koe, Salzberg, Morris, O'Brien, & Jones, 2014; Villaseñor-Garcia et al., 2001; Viveros-Paredes et al., 2006). We have studied the effect of acute and chronic stress on activation of the HPA axis using a model of electrical stimulation (ES, physical stress) and LPS administration (immune stress), evaluating the increase produced in the levels of corticosterone and pro-inflammatory cytokines. Corticosterone levels were increased by chronic stress and by acute immune stress, but not by acute physical stress. Indeed, it is known that during acute stress the amplitude and synchronization of CRH and arginine-vasopressin (AVP) pulsations in the hypophyseal portal system increases markedly, resulting in increases in the shortest episodes of ACTH and cortisol secretion (Hueston & Deak, 2014; Tsigos & Chrousos, 1994; Wallon & Soderholm, 2009). Therefore more time points might be necessary to determine whether ESa was actually capable of enhancing the levels of corticosterone.



On the other hand, after mice were exposed to an acute or chronic challenge with LPS, the plasma levels of corticosterone increased. These data are in agreement with previous studies where single injections of LPS cause marked activation of the HPA axis with the ensuing increases in hypothalamic CRH, plasma ACTH and corticosterone levels (Girard-Joyal et al., 2015; Grinevich et al., 2001; Quan, Whiteside, & Herkemham, 1998; Rivest, Laflamme, & Nappi, 1995; Turnbull & Rivier, 1999). Repeated LPS injection led to chronic activation of the HPA axis as shown by the increase in CRH activity on the PVN and on pituitary pro-opiomelanocortin (POMC) mRNA expression 24 h after the last injection (Takemura et al., 1997). However, the elevated corticosterone levels in response to repeated LPS administration suggests an alteration of the regulatory mechanisms that are responsible for controlling the HPA axis. The responses to chronic stress are at times somewhat contradictory. While some studies have shown diminished sensitivity to fast feedback signals following chronic stress (Hutton et al., 2015; Jaferi, Nowak, & Bhatnagar, 2003; Mizoguchi, Ishige, Aburada, & Tabira, 2003; Young, Akana, & Dallman, 1990), others have shown that chronically stressed animals display enhanced delayed feedback inhibition when compared to acutely stressed animals (Gadek-Michalska, Szyrka, Rachwalska, Tadeusz, & Bugajski, 2013; Mizoguchi et al., 2001; Silverman & Sternberg, 2012).

In relation to the activation of the HPA axis and of pro-inflammatory cytokines, we found that TNF- $\alpha$  levels were increased following ESc. This is consistent with the observation that the stress-induced rise in corticosterone masks a robust and widespread increase of TNF- $\alpha$ .

With regards to acute LPS stimulation, the levels of pro-inflammatory cytokines increased due to the activation of the HPA axis in response to this peripheral immune challenge (Eskilsson et al., 2014; Johnson, O'Connor et al., 2002; Kanczkowski, Sue, Zacharowski, Reincke, & Bornstein, 2015; Nandi, Mishra, Basu, & Bishayi, 2010). In agreement with previous data, a single injection of LPS produces a considerable increase in TNF- $\alpha$  as well as in IL-1 and IL-6 within 90 min (Hansen et al., 2000; Kapcala, He, Gao, Pieper, & DeTolla, 1996; Lenczowski, Van Dam, Poole, Larrick, & Tilders, 1997). Likewise, we demonstrate here that mice exposed to an acute LPS challenge experienced an increase in TNF- $\alpha$ , IL-1 $\beta$  and IL-6 cytokine levels 90 min after i.p. injection of 250  $\mu$ g/100 g. These results suggest that IL-1 $\beta$  may directly stimulate primary sensory fibers of the vagal nerve to activate the HPA axis after i.p. LPS administration (Kapcala et al., 1996; Lenczowski et al., 1997) and this activation depend IL-1 type 1 receptors (IL-1R1)(Matsuwaki, Eskilsson, Kugelberg, Jonsson, & Blomqvist, 2014). However, chronic LPS administration had no effect on either TNF- $\alpha$  and IL-1 $\beta$  levels, perhaps due to the high corticosterone and IL-10 levels

(Castellucci et al., 2015; Villaseñor-Garcia et al., 2001; Viveros-Paredes et al., 2006), and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) levels (Wei et al., 2015). In this sense, LPS not only induces an increase in pro-inflammatory cytokines but also in anti-inflammatory cytokines such as IL-4, IL-10, IL-13 and transforming growth factor- $\beta$  (TGF- $\beta$ ). Anti-inflammatory cytokines have the ability to suppress the synthesis of IL-1  $\beta$ , TNF- $\alpha$ , and other cytokines such as IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Furthermore, they can upregulate the expression of the IL-1 receptor antagonist (IL-1ra) (Bluthé et al., 1999; Kremlev & Palmer, 2005; Vasseur et al., 2014). Similarly, IL-6 levels also augmented following chronic LPS administration and this increase may be related to the elevated levels of corticosterone found in this study. This cytokine is involved in the activation of the HPA axis in response to repeated LPS injection, and also sensitized the induction of hypothalamic IL-1 $\beta$  in response to footshock stress (Girotti, Donegan, & Morilak, 2011). Moreover, IL-6 can stimulate the production of leukemia inhibitory factor (LIF), induces expression of the ACTH precursor (proopiomelanocortin) and secretion of ACTH in primary corticotrophs or cell lines, this effect being synergistic with CRH (Nicola & Babon, 2015).

## Conclusion

Taken together, the present results suggest that chronic electrical stress activates the HPA axis and increases both corticosterone levels as well as plasma TNF- $\alpha$  levels. Moreover, acute or chronic i.p. administration of LPS induces activation of the HPA axis and this activation leads to an increase in corticosterone levels, suggesting that this stimulus affects the regulatory mechanism responsible for controlling the HPA axis. On the other hand, the cytokines IL-1 $\beta$  and IL-6 are affected by the activation of the HPA axis, suggesting that IL-6 might also be involved in the changes in the pituitary and adrenal secretory response related to corticosterone release at the systemic level. Thus, the response to the activation of the HPA axis by stress depends on the duration, intensity and etiology of the stress agents involved.

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