The Effects of Cortistatin Administration on Plasma Antioxidant System and Cytokine Levels of Rabbits with Acute Inflammation

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ABSTRACT

In the study, it was aimed to research the effects of an endogenous neuropeptide called cortistatin (CST) which is isolated from the brain on parameters of antioxidant system and cytokine levels of rabbits with turpentine oil induced acute inflammation. In the study using 28 healthy, male rabbits from New Zealand breed, animals were randomly divided into four groups consisting of control (C), turpentine (T), cortistatin (250 µg/kg) (CST), and turpentine + cortistatin (250 µg/kg) (T+CST). Reduced glutathione (GSH), super oxide dismutase (SOD, malondialdehyde (MDA) levels belonging to antioxidant and oxidant systems, interleukin-6 (IL-6) and tumor necrosis factor -α (TNF-α) levels from inflammatory cytokines were studied in the blood samples taken after 2 and 6 hours and while cortistatin administration significantly contributed for maintaining GSH at protective level in T+CST group ($p<0.05$), it caused a change which was not statistically significant with respect to MDA and SOD levels increasing based on elevated lipid peroxidation levels in the tissue along with the inflammation ($p>0.05$). Depending on resulting data of the study, it was concluded that despite there were effects observed regarding that cortistatin plays an inflammation suppressing role, this was smaller than the effect observed in conditions such as endotoxemia and sepsis because design of study did not lasted for long.

Key words: Cortistatin, antioxidant parameters, reduced glutathione, malondialdehyde, interleukin-6.

Akut Enflamasyonlu Tavşanlarda Kortistatin Uygulamasının Antioksidan Sistem ve Sitokin Seviyeleri Üzerine Etkileri

ÖZ

Çalışmada, beyinden izole edilen kortistatin (CST) isimli endojen bir nöropeptidin, terebentin yağı ile akut enflamasyon oluşturulan tavşanlarda antioksidan sistem parametreleri ve sitokin düzeyleri üzerine etkilerinin incelenmesi amaçlanmıştır. Sağlıklı, 28 adet erkek Yeni Zelanda ırkı tavşan kullanılan araştırmada, hayvanlar kontrol (C), terebentin uygulaması (T), kortistatin uygulaması (250 µg/kg) (CST) ve terebentin + kortistatin (250 µg/kg) (T+CST) uygulaması olacak şekilde rastgele dört gruba ayrılmıştır. 2. saatte ve 6.saatte alınan kan örneklerinden antioksidan ve oksidan sistemlere ait indirgenmiş glutatyon (GSH), süper oksit dismutaz (SOD) ve malondialdehit (MDA) düzeylerinin, enflamatuar sitokinlerden ise interlöykin-6 (IL-6) ve tümör nekrozis faktör -α (TNF-α) seviyelerinin incelendiği araştırmada kortistatin uygulaması, T+K grubunda GSH seviyesinde T grubuna nazaran anlamlı derecede koruyucu seviyenin sürdürülmesine katkıda bulunurken ($p<0.05$), enflamasyonla birlikte dokuda lipid peroksidasyon düzeyinin artışına bağlı olarak yükselen MDA seviyesi ve SOD seviyeleri açısından ise istatistiki olarak önemli olmayan bir değişikliğe yol açmıştır ($p>0.05$). Çalışmanın sonucunda elde edilen verilere dayanarak kortistatin, enflamasyonu baskılayıcı bir rol oynadığına dair etkiler gözlenmesine rağmen, çalışma dizaynı kısa sürdüğü için, bunun endotoksemi ve sepsis gibi durumlarda gözlenen etkisinden küçük olduğu sonucuna varılmıştır.

Anahtar Kelimeler: Kortistatin, antioksidan parametreler, redükte glutatyon, malondialdehit, interlöykin-6.

1. INTRODUCTION

Cortistatin (CST) which is a cyclic neuropeptide discovered in recent years has been under the spotlight for utilizing in terms of efficiency in various cases that are inflammatory, endocrinological, neurophysiologic, etc. Peptide has been reported to be expressed particularly from cortical region of the brain and to suppress cortical activity (de Lecea et al., 1996). While expression of cortistatin, earlier, was accepted to be limited with rat cerebral cortex and hippocampus (Rubinfeld and Shimon, 2007), today it is known to have a wide distribution in numerous peripheral tissues such as retina, adrenal gland, thyroid gland, pancreas, testis, liver, stomach, ileum, jejunum, colon, rectum, kidney, lung, parathyroid gland, and immune system (Marchenko and Strongin, 2001: Baranowska et al., 2006: Carrasco et al., 2008). Although many of the roles attributed to cortistatin are suggested to be within the scope of cytokine functions, they are not given inside this classification because they exist at low levels in the body.

Cortistatin is described as a strong anti-inflammatory substance which can deactivate inflammatory response in vivo (Broglio et al., 2007: Rubio et al., 2007) and also has a neuroprotective agent activity (Wilson et al., 2005: Marcovicks et al., 2012: Morell et al., 2013). Cortistatin having high degree of homology with somatostatin (SST), shares a large number of pharmacological and functional characteristics with somatostatin by binding with five cloned somatostatin receptor (SSTR1-5). These functional characteristics include depression of neuronal activity and inhibition of cellular proliferation (de Lecea et al., 1996).

Inflammation; is a protective host response that occurs against penetration of an foreign antigen in the body or tissue damage, can cause structural deformation of tissue as well as dysfunction unless it is treated. The fact that most inflammatory processes are self-restricted and self-reactivated suggests the likelihood for the existence of endogenous anti-inflammatory or pro-resolution mediators in the course of inflammation (Lawrence et al., 2002: Gilroy et al., 2004: Serhan et al., 2004).

Once immune system is triggered by any stimulus, cooperation between natural and adaptive immunity results in activation of different cell types and immunological molecules that collaborate in order to eliminate the damage (Janeway and Medzhitov, 2002). Both stress and immunological stimulus activate various neuron groups triggering certain crucial molecular pathways that are found in the hypothalamus and generally have anti-inflammatory and immunosuppressive characteristics for limiting immune response (Steinberg, 2006).

The fact that plasma concentrations of acute phase (AP) proteins do not increase under several stress conditions as an example (Poznanovic et al., 1997: Gabay and Kushner, 1999), suggests that inflammatory mediators are regulated by specific suppressors (Engler, 1995). For long years, pre-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin 1-β (IL1-β) have been known to be the most critical mediators with effect on production of AP proteins in the liver (Alsemgeest et al., 1996: Yoshioka et al., 2002).

Some researchers proposed that this peptide could have a major endogenous immune system regulatory function in the immune system (Rubinfeld and Shimon, 2007), in addition could also bind

receptors of anti-inflammatory mediatory hormones such as ghrelin which is a secretagog of growth hormone (Deghenghi et al., 2001: Granado et al., 2005: Lugue et al., 2006).

For our study in which started in order to evaluate immune system associated characteristics of cortistatin from those mentioned above, we preferred subcutaneous turpentine administration as a model of acute inflammation because injections of oleoresin from Pinus pinaceae, that is turpentine oil, are commonly used model for inductor of the acute phase response in laboratory animals (Baumann et al., 1984). Previous research reported that nonspecific host response to inflammation was widely mediated with the release of pro-inflammatory cytokines such as IL-1, TNF- α and IL-6 (Gabay and Kushner, 1999). Furthermore, prevention of IL-1 to bind type-1 receptor (Gershenwald et al., 1990: Oldenburg et al., 1995) or IL-6 inhibition by monoclonal antibodies were reported to prominently attenuate inflammatory response to turpentine inflammations. Similar results were also obtained with rats for IL1-β, type-1 IL-1 receptor and IL-6 knockout (Fantuzzi and Dinarello, 1996: Labow et al., 1997). Hepatic acute phase response induced against anorexia, weight loss, turpentine-indüced inflammations was notified to be mediated by response of endogenous IL-6 which starts with IL-1 binding to type-1 receptor (Sims et al., 1993). Due to all these facts, we determined IL-6 and TNF- α, being representatives of pro-inflammatory cytokine, as serum parameters.

When all these above addressed together, these results show that neuropeptides can target a wide spectrum of inflammatory mediators regulating immune response at different levels. This is likely to be the most advantageous effect of neuropeptide treatment on current therapies.

In the light of foresaid literature information, it was aimed in the current study to examine the effects of cortistatin administration on certain antioxidant system parameters and pro-inflammatory cytokines in inflammation which was subcutaneously induced by turpentine injection.

2. MATERIAL AND METHOD

New Zealand white breed rabbits which are 8-12 months old, 1.5-2 kg weighed 28 healthy, and male, were used in the research. Animals were weighed and randomly assigned to 4 groups including Control Group (C), turpentine Group (T), Cortistatin Group (CST), and Turpentine + Cortistatin Group (T+CST) which were close in mean group weight. For sheltering all of the animals, individual rabbit cages made of stainless steel and containing fixed feeder, watering and pan were used and while animals were ad libitum fed with commercial rabbit feed (MBD feed) with composition prepared according to measurements prescribed by Nutrient Requirements of Rabbits (NRC, 1977) and its content was given in Table 1, water was always provided fresh.

Table 1. *Composition of commercial rabbit feed and its analysis*

Content	Amount %
Dry matter	89.4 %
Crude protein	18.0 %
Crude cellulose	14 %
Crude ash	6.45 %
Crude lipid	4.5 %
Calcium	1.0 %
Total phosporus	0.59 %
Lysine	1.0 %
Methionine	0.60 %
Methionine-cystine	0.90 %
Natrium	0.27 %
Linoleic acid	1.26 %
Metabolic energy	2650 calori/kg

Cortistatin-29 (rat) Trifluoroacetat which would be applied was provided from (H-6444, Bachem AG) Bachem company, Turpentine (Oil of turpentine, 24245-Aldrich) from Sigma- Aldrich company. 5 ml normal saline solution in total, 2.5 ml for the right and 2.5 ml for the left paravertebral region, was injected to 7 rabbits assigned in the control group. 250 µg/kg cortistatin in dose was administered intraperitoneally to animals in CST group. Five ml turpentine oil, 2.5 ml for the right and 2.5 ml for the left paravertebral region, was injected subcutaneously to animals in T group in order to induce acute inflammation. For the animals in CST+T group, on the other hand, 5 ml turpentine oil, 2.5 ml for the right and 2.5 ml for the left paravertebral region, was injected subcutaneously 30 minutes after intraperitoneal administration of 250 µg/kg dosed cortistatin.

Blood samples from all animals were drawn in to blood collection tubes with K EDTA and heparin appropriately with the purposes of measurement after 2 and 6 hours from the initiation of the experiment. Samples taken in the intermediate period were from auricular vena (V.auricularis magna), the last blood sample was drawn through intra-cardiac puncture from animals under ketamine-xylazine anesthesia. Following the last drawn blood samples, all animals were subjected to euthanasia via cervical dislocation. Collected samples were immediately centrifuged with Hettich Zentrifugen, Rotina 380 R brand cooled centrifuge at 3000 rpm for 15 minutes, apportioned in to eppendorfs and conserved at -20ºC until analyzed.

Superoxide dismutase enzyme was measured in BioTek ELx800TM microplate reader using Cayman Chemical SOD Assay Kit. Level of malondialdehyde (MDA) which is from lipid peroxidation products was measured using thiobarbituric acid activity method (Akkuş, 1995), reduced glutathione level, according to method by Beutler et al., (1963) by measuring absorbance of specimen and standard versus blank in PG Instruments T807T80 +UV-Vis UV-VIS spectrophotometer at 412 nm wavelength (Beutler et al., 1963).

Data obtained from all groups in the research were evaluated by the aid of SPSS 10.0 package (SPSS, Inc., Chicago, IL) software, by performing variance analysis for determining the significance of differences between groups at the same sampling period belonging to all data via Duncan's Multiple Range test, student t test for determining the significance differences between sampling times. For comparisons, >0.05% of difference was accepted statistically significant.

3. FINDINGS

MDA level, measurements of antioxidant system parameters and cytokine levels obtained from all groups were given in Table 2 and Table 3.

Table 2. *Results of antioxidant system parameters determined following cortistatin administration (X±SEM, n=7)*

Groups	n (28)	MDA (nmol/ml erythrocyte)		GSH (mg/dl)		SOD (U/ml)	
		2nd hour	6th hour	2nd hour	6th hour	2nd hour	6th hour
C	**7**	3.89±0.43	3.67±0.29 a	34.23±0.04	32.21±0.67 a	0.295 ±0.67	0.302±0.89 a
T	**7**	3.60±0.57 A	4.89±0.87 Bb	33.45±0.98A	28.89±0.65Bb	0.287±0.73 A	0.262±0.92 Ba
CST	**7**	3.65±0.73	3.9±0.22 a	35.11±0.87A	32.69±0.46ABa	0.301±0.56	0.291±0.40 a
T+CST	**7**	3.89±0.43 A	4.21.±0.43Bab	31.57±0.90	34.23±0.04 a	0.288 ±0.92	0.270±0.67 ab

AB: Difference between sampling times with different letters in the same line is significant (P<0.05)

ab: Difference between groups with different letters in the same column is significant (P<0.05)

Table 3. *Results of plasma TNF-α and IL-6 levels determined after cortistatin administration (X±SEM, n=7)*

Groups	n (28)	TNF-α (pg/ml)		IL-6 (pg/ml)	
		2nd hour	6th hour	2nd hour	6th hour
C	**7**	12.18±0.43 a	13.23±0.78 a	24.72±0.09	22.87±0.07a
T	**7**	16.16±0.24Ab	24.09±0.87 Bb	26.41±0.01B	35.89±0.11Bb
CST	**7**	12.18±0.43 a	11.67±0.90 a	25.29±0.47	24.76±0.94a
T+CST	**7**	12.18±0.43 a	14.12±0.71 a	27.77±0.09	26.92±0.32a

AB: Difference between sampling times with different letters in the same line is significant (P<0.05)

ab: Difference between groups with different letters in the same column is significant (P<0.05)

4. RESULTS AND DISCUSSION

Neuropeptides are small and hydrophilic molecules with the ability to pass in to inflammation area fast, can be rapidly eliminated from the body through natural hepatic detoxification mechanisms and renal excretion. Therefore, their half-life and action time are relatively last short. In addition, other cytokines, neuropeptides, and hormones balance the impacts of neuropeptides and thus, homeostasis of normal tissues is not disrupted. Despite all these advantages, there are certain obstacles in transferring neuropeptide based treatments to live clinical practices. First of all, owing to their peptide-like structure, they are not highly stabile and tremendously susceptible to peptidases that are readily

found in the most tissue. For this reason, pharmaceutical companies currently focus on developing metabolically stabile analogues of these peptides.

Understanding these neuropeptides and their specific receptor, structure-function relationship such as receptor signalization, internalization, homo and hetero-dimerization in physiological and pathological conditions will make it easy to develop novel pharmaceutical agents. It has been also focused on strategies which allow to develop new strategies enabling direct use of natural neuropeptides, increased half-life of neuropeptide, and particularly their increased distribution in targeted tissue during long term treatments (Souza-Moreira et al., 2011).

Earlier, while it was focused on interaction of cortistatin with nervous system and endocrine system which have close anatomic relationship with cortistatin, now there are also studies regarding its impacts on immune system.

Chiu et al., (2011) determined that endogenous prepro-CST mRNA expression increased in parallel with the condition of inflammation in a rat model with *Klebsiella pneumonie* bacterium induced encephalitis and interestingly this also occurred before infiltration of leucocytes. Intracerebroventricular or intraperitoneal administration of CST following the infection decreased leucocytes to accumulated in the region and clinical disease view occurring with fewer and clinical score (Chiu et al., 2011). In our study, even though a decrease was also determined in IL-6 cytokine levels as the result of intraperitoneal CST administration within in a very short time approximately half an hour later turpentine injection of acute inflammation model, it will be beneficial to examine clinical findings and tissue pathology in studies that will be conducted for a better understanding of the issue.

Gonzales-Rey et al., (2006) determined CST to inhibit production of TNF-α, IL-6, and nitric oxide from inflammatory mediators of in vitro activated macrophages. In rats injected with different doses of lipopolysaccharides, it was suggested that CST administration in 2 nmol/rat (250 μg/kg) of dose about 30 minutes after LPS administration protected against lethal dose of LPS and this protective effect was dose-depended, was relatively preservative even at the minimum dose (0.5 nmol/rat, 50 μg/kg). CST administration improved the ratio of survival in experimentally induced sepsis model, decreased local and systemic anti-inflammatory cytokines (TNF-α, IFN-γ, IL-6, IL1-β and IL-12), chemokines (MIP-2, RANTES) and NO levels and systemic measurements of acute phase proteins such as serum amyloid A (SAA) level, prevented histopathology (inflammatory cell infiltration, development of Disseminated Intravascular Coagulopathy in several organs) observed in septic shock.

In 2006, Leavy induced formation of colitis (a model similar with the clinical, pathological, and immunological characteristics of human Crohn's disease) in rats by 2,4,6 trinitrobenzene sulfonic acid (TNBS) in their study, administration of CST after 12 hours was found to protect these animals

against formation of colitis. Administration of CST also recovered clinical symptoms and histopathology of colitis induced by TNBS, such as weight loss, diarrhea, intestinal inflammation, and mortality. Surprisingly; administration of CST 6 days after TNBS administration eliminated the relevant disorder and was also effective about decreasing recurrence ratio of the disorder. With the analysis of inflammatory mediators in colon, on the other hand, in study indicating that there was a partial decrease in production of several proinflammatory cytokine and chemokine in rats administered with CST, a decrease was also observed in inflammatory cell infiltration in colonic mucosa. Decrease in production of cytokine and chemokine was not as a result of decrease in the number of cells infiltrating to region, was due to direct suppression of lamina propria mononuclear cells. In addition, CD4+T cells isolated from CST administered rats with colitis produced γ-interferon lesser than T cell of rats without administration (Leavy, 2006).

Again, administration of CST increased the production of anti-inflammatory cytokine IL-10. However, while the number of CD4+T cells producing γ-IFN isolated from lamina propria decreased following CST administration, the number of IL-10 producing cells increased. Depending on observations in this study, it was suggested that CST induced activation and/or formation of T cells producing IL-10, with this it could be involved in protective effect against development of colitis. In another study, protective role of CTS was shown in septic shock model. CST administration protected rats against endotoxin induced lethality and histopathology. These effects were seen to be through suppression in production of proinflammatory cytokine, chemokine and acute phase protein. Administration of CST, at the same time, increased the production of IL-10 in this model. These data indicates that CST suppressing production of proinflammatory mediators by increasing production of IL-10 in two different disease model is a strong anti-inflammatory agent. Therefore, authors speculated that CST could be presented a strong therapeutic agent for Crohn's disease and septic shock (Leavy, 2006).

Combined administrations of cortistatin with vasoactive intestinal peptide which is an anti-inflammatory endogenous peptide substantially prevented lethality induced by high dose of LPS and it was emphasized that therapeutic dose could be decreased with such combined administrations (Gonzales-Rey et al., 2006).

In their retinal study, Carrasco et al., (2008) introduced that tumor necrosis factor -α (TNF-α) included in diabetic retinopathy and low CST levels could be as the result of this inflammatory condition. In study by Yan et al., (2005) carried on human coronary endothelial cells, TNF-α displayed a strong down regulation on CST expression.

Exogenous pyrogens such as turpentine, amphetamines, and sulfur were reported to cause fewer by inducing the release of proinflammatory cytokines such as IL-1, IL-6 and TNF-α which are assumed as endogenous pyrogens from macrophage and monocytes (Koj, 1985: Soszynski and Krajewska, 2002). In our study, administration of turpentine to animals in inflammation group (T)

increased plasma IL-6 and TNF-α levels compared to control group, this a condition which is compatible with the literature and expected.

Likewise, cortistatin administration in treatment of collagen induced arthritis model was shown to down regulate production of TNF-α, IFN-γ, IL-6, IL1-β and IL-12 in inflamed joints, to trigger a simultaneous increase of IL-10 and TGF-β levels which are anti-inflammatory cytokines (Gonzales-Rey et al., 2007).

The effect of TNF- α blockage on inflammatory response to turpentine abscess is one of the controversial issues. Some researchers could not observe any difference in response to inflammation of turpentine abscess in TNF receptor knockout rats or TNF-α antibody administered rats (Gershenwald et al, 1990: Leon et al., 1997). Tous et al., (2005) injected turpentine-olive oil mixture (1:1) to hind leg twice a week for 12 weeks in order to induce chronic aseptic inflammation model in rats by turpentine. They determined increase in concentrations of TNF-α and serum amyloid A (SAA) which are indicator of systemic inflammatory response in turpentine administered rats.

Increase of serum TNF-α and serum amyloid levels in rats that were chronically administered with turpentine for two weeks was interpreted to indicate systemic inflammatory response induced by turpentine (Tous et al., 2005). Injection of turpentine increases TNF-α levels after 6 hours, this duration is a time of period when cytokine progress at known peak level.

It was showed that CST inhibited production of proinflammatory cytokines and nitric oxide released from activated macrophages and influenced levels of these molecules in blood stream and stimulated release of anti-inflammatory cytokine interleukin-10 appropriate to dramatic increase of survival and clinical result in rat models' sepsis, Crohn's disease, and rheumatoid arthritis (Gonzales-Rey et al., 2006a: Gonzales-Rey et al., 2006b: Gonzales-Rey et al., 2007). Being compatible with mentioned research, we observed the statistically significant decrease of proinflammatory cytokine IL-6 level after 6 hours we measured in our study as well ($p<0.05$).

Increase of IL-6 level in T group of our study seems to be compatible with the study in which maximum (12.5 times) increase of serum IL-6 concentration in rats after 6 hours (Sheikh et al., 2006). In both studies TNF-α concentration remained the same. The most likely reason of this result is that circadian rhythms of cytokines are different.

Extremely toxic hydroxyl radical covalently binds to proteins and carbohydrates, leads to lipid peroxidation, and catabolizes the cell membrane. Measurement of lipid peroxidation is a conventional method used in examination of oxidative damage (Viani et al., 1991).

When obtained findings were evaluated considering the difference between sampling times, while there was not a significant difference between data from control group and cortistatin, statistical significance was observed only in TNF-α level of turpentine group ($P<0.05$).

Consequently, it could be speculated that while injection of cortistatin tested for protective purposes in rabbits with induced acute septic inflammation had both IL-6 and TNF-α levels close to control group, there was statistically significant difference only on GSH levels from parameters of antioxidant system, this likely could be resulted from the fact that experiment ended after 6 hours.

The current study which we had to limit with 6 hours in procedure is more likely to be the possible reason of certain effects of cortistatin which could not be determined as statistically significant. Therefore, we recommend to design studies with data observed after 24 hours, even after 48 hours, in order to observe the impacts clearly.

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