Neuroprotective Effects of Food Restriction in a Rat Model of Traumatic Brain Injury - The Role of Glucocorticoid Signaling

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Running title: Diet, glucocorticoids and brain injury

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Abstract

Traumatic brain injury (TBI) is one of the most common causes of neurological damage in young and middle aged people. Food restriction (FR) has been shown to act neuroprotectively in animal models of stroke and TBI. Indeed, our previous studies showed that FR attenuates inflammation, through suppression of microglial activation and TNF-α production, suppresses caspase-3-induced neuronal cell death and enhances neuroplasticity in the rat model of TBI. Glucocorticoids (GCs) play a central role in mediating both molecular and behavioral responses to food restriction. However, the exact mechanisms of FR neuroprotection in TBI are still unclear. The goal of the present study was to examine whether FR (50% of regular daily food intake for 3 months prior to TBI) exerts its beneficial effects by altering the glucocorticoid receptor (GR) signaling alone and/or together with other protective factors. To this end, we examined the effects of FR on the levels of total GR, GR phosphoisoform Ser^{232} (p-GR) and its transcriptional activity, as well as 11β-HSD1, NFκB (p65) and HSP70 as factors related to the GR signaling. Our results demonstrate that FR applied prior to TBI significantly changes p-GR levels, and it's transcriptional activity during the recovery period after TBI. Moreover, as a pretreatment, FR modulates other protective factors in response to TBI, such as 11β-HSD1, NF-κB (p65) and HSP70 that act in parallel with GR in it’s anti-inflammatory and neuroprotective effects in the rat model of brain injury.

Keywords: Brain injury, Food restriction, Glucocorticoids, Neuroprotection

1. Introduction

Different dietary paradigms in rodent models have provided consistent support for the hypothesis that a decrease in calories consumed (up to 50%) sustain both neuronal viability and neuroprotection when faced with an insult such as stroke or exposure to neurotoxins[1]. Among several hypotheses about the underlying mechanisms triggering neuroprotective effects of food restriction (FR), the prevalent one points to FR as a mild stressor, protecting neurons against subsequent severe stress conditions [2]. In that regard, several data demonstrated a central role for elevated GCs in mediating both molecular and behavioral responses to FR[3] with these findings considered paradoxical, since a prolonged increase in GCs is commonly associated with the brain damage[4]. This led to a hypothesis that the elevated GCs provide neuroprotection by activating a network of stress-protective pathways[5].

Traumatic brain injury (TBI) is a major health and socio-economic problem worldwide[6]. It is a complex pathological process comprised of two phases, primary and secondary injury. Primary injury is the result of immediate mechanical damage to the brain tissue, while the secondary injury is characterized by a set of biochemical cascades that inevitably leads to the death of neighboring cells not initially affected by the injury[7]. The main feature of secondary injury is enhanced immune response reflected by the presence of inflammatory cells[8] and proinflammatory cytokines around the injured area that further accelerates the process of secondary brain injury[9]. Synthetic glucocorticoids (GCs) were widely used as a therapy to reduce secondary injury following TBI[10], attenuating the inflammatory response. However, large-scale clinical trials of GCs, as a

treatment for TBI, have either failed to demonstrate efficacy or even revealed an increased risk of mortality[11,12].

Our previous studies have demonstrated that FR, applied before TBI, significantly increases circulating GC - corticosterone (CORT) levels in rats, while at the same time it attenuates inflammation, through suppression of microglial activation and TNF-α production, suppresses caspase-3-induced neuronal cell death and enhances neuroplasticity in the injured brain[13,14,15]. The goal of the present study was to elucidate whether the FR-induced serum CORT elevation leads to changes in GR signaling in the injured brain. We also aimed to clarify whether FR exerts its beneficial effects by altering only GR signaling, or it activates other protective factors that by acting together with GR, lead to previously observed enhanced neuroprotection and abolishment of inflammation. To address this, we examined the effects of FR on the levels of total GR, levels of GR phosphoisoform Ser^{232} (p-GR) and its transcriptional activity, as well as 11β-HSD1, NFκB(p65) and HSP70 as factors related to the GR signaling.

2. Materials and Methods

2.1. Animals

Adult male Wistar rats (3-month-old at the beginning of experiment) were housed under standard conditions (23+2°C, 60–70% relative humidity, 12h light/ dark cycles, with the lights switched on at 07:00; free access to water; n=3 per cage). All animal procedures were approved by the Committee for Ethical Animal Care and Use of the Institute for Biological Research, University of Belgrade (Permit Number: 26/06), which acts by the Directive (2010/63/EU) on the protection of animals used for experimental and other

scientific purposes. Minimal numbers of animals was used and all efforts were made to minimize animal suffering.

2.2. Food Restriction

Daily food intake for each animal was measured for five consecutive days before the beginning of the experiment, and the average daily food intake was estimated at 24 g of standard laboratory chow pellets/day/rat (Table 1).

The AL group had unlimited access to food during the entire experiment. Animals from the FR group were fed 50% of their normal daily food intake (12 g/day/rat); food was provided at 12:00. Both groups of animals had ad libitum access to water. Considering that the food demand increases during animal life, the daily food intake was measured in the AL group once a month in order to assure that the correct amount of food was provided to the FR rats. At the end of the experiment, the daily food intake in the AL group was estimated at 28 g food/day/rat. Consequently, in the FR group the daily food intake was 14 g/day/rat. The body weight of AL and FR rats was measured every other week. As we previously published, at the time of surgery the animals in the FR group weighed about 30% less than the AL animals[13]. Rats in the FR group were maintained on a restricted feeding regimen during the entire period of recovery after the surgery.

2.3. Experimental Procedure

Animals from both AL and FR groups were randomly divided into five groups according to the duration of the recovery period, i.e. 2, 7, 14 and 28 days post-injury (dpi) (n=8 per group). Surgery was performed when the rats reached 6 months of age, as previously described[13].

Briefly, the animals were anesthetized with Nembutal (50 mg/kg, Serva) and positioned into a stereotaxic frame and the scalp was shaven. An incision was made along the midline of the scalp and the Bregma was exposed. Cortical focal injury was performed unilaterally using 1 mm wide dental drill through the left somatosensory cortex. The drill was inserted 1 mm into the cortex. After surgery, the animals were kept in isolation for 3 h until complete recovery from the anesthesia, and subsequently returned to their home cages in order to avoid social isolation stress (n= 3 per cage). At the end of the recovery period, the animals were decapitated, the brains were removed on ice, and the ipsilateral cortex was collected from each animal for subsequent analysis. Physiological controls (designated as control) for both groups were not subjected to surgery. Cortical tissue from the same animal was used for RNA and protein isolation (n=5 animals per each time point). For histochemical analysis, entire rat brains (n=3 animals per each time point) were used. The presented results denote the data of three independent experiments.

2.4. RNA Isolation, Reverse Transcription, and Semiquantitative RT-PCR

Total RNA from ipsilateral cortical tissues was isolated and reverse transcription and semiquantitative RT-PCR were done as previously described[13]. For serum and glucocorticoid-regulated kinase 1 (Sgk-1) mRNA real-time RT-PCR analysis, the rat Assays-on-Demand Gene Expression Product (Applied Biosystems) was used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a reference gene. Validation experiments were performed as previously described[16]. The obtained results (expressed as fold changes) were tested for statistical significance ($p<0.05$) using the RQ Study Add ON v 1.1 software (Applied Biosystems).

2.5. Western Blot Analysis (WB)

For WB, total proteins from ipsilateral cortical tissues were isolated. Tissue isolation, sample preparation, SDS acrylamide gel electrophoresis, blotting onto nitrocellulose membrane and membrane blocking were done as previously described[13]. The membranes were incubated with primary antibodies (Table 1) overnight at 4ºC.

The membranes were then incubated with the appropriate HRP labeled secondary antibodies: anti-rabbit antibody (1:2000; sc-2370, Santa Cruz, USA) and anti-goat antibody (1:5000; sc-2350, Santa Cruz, USA) in TBST for 1 h at room temperature. The signal was detected by enhanced chemiluminescence (ECL, Amersham Bioscience) and exposure of an X-ray film (Kodak). All films were analyzed by densitometry using the image analysis program ImageQuant ver.5.2 (Amersham). Each blot was subsequently re-probed with rabbit anti-actin antibody (1:10000; Santa Cruz Biotechnology, USA) in TBST, overnight at +4C° that served as endogenous control. The membranes were incubated with the HRP-labeled secondary anti-rabbit antibody (1:2000; sc-2370, Santa Cruz, USA) in TBST for 1 h at room temperature. The levels of the target protein were determined relative to the appropriate control values in AL rats that were set as 100%.

2.6. Immunohistochemical Analysis (IHC)

For IHC analysis, rat brains were prepared as previously describe[14]. The sections were incubated in PBS overnight at 4 °C with rabbit polyclonal anti-Ser211 GR antibody (1:50, Cell Signaling #4161S), and after rinsing, with the AlexaFluor-555 labeled goat-anti-rabbit IgG (Invitrogen). Following counterstaining with SYTOX® green fluorescent nuclear and chromosome nucleic acids staining, the sections were mounted in fluorescent

mounting medium (Dako). To test the specificity of the immunolabeling, the primary antibody was omitted in control experiments. All the photomicrographs were obtained using Leica TCS SP5 Confocal Laser Scanning Microscope (Leica Microsystems) equipped with Argon 488 nm and NeHe543/633 nm lasers using 20x/1.30 lens. Emission of fluorescently labelled p-GR and SYTOX® green were collected sequentially. All images were taken with a 512×512 pixel resolution and 8-bit color depth. For the purpose of colocalization analysis three sections per animal, from three animals were used. The tissue that surrounds lesion (in the zone of 200μm from the lesion border) was used as the region of interest for further analysis. Colocalization analysis was performed in ImageJ software (NIH, USA) and the degree of colocalization between p-GR and SYTOX® green was measured using the Pearson correlation coefficient (PCC). PCC measures the strength of a linear relationship between fluorescent intensities from the two images and produces values ranging from 1 (perfect positive correlation) to -1 (perfect inverse correlation), with 0 representing a random distribution[17].

Analyses of p-GR-positive cells number and number of SYTOX green cells were done using Image J with threshold processing (Renyi Entropy) and low surface area limit set to 3μm2 for p-GR^{Ser232} and 5μm2 for SYTOX green immunostaining. The low surface area limit was set to exclude artefact in number of cells.

2.7. Statistical Analysis

Data were analyzed by two-way analysis of variance (ANOVA) with feeding regiment and time as factors. Post hoc comparisons were made with Fisher's LSD test. Comparisons with appropriate control values for each group were made using the t-test.

Mutual correlations on the protein p-GR/NFκB(p65)/HSP70 expression levels and their association with pathological features after the TBI in cortical tissue were analyzed using Pearson correlation test, prior to which normality test was applied. The degree of correlation was determined using Pearson correlation coefficient and R square. All statistical analysis was performed using GraphPad Software (San Diego, CA). All data were expressed as mean ± SEM. P value differences were considered to be statistically significant when $p<0.05$.

3. Results

At the age of six months, both groups of animals AL (normally fed during entire the experiment) and FR (under 50% reduction of normal daily food intake since 3rd month of age) were subjected to unilateral focal lesion of the left somatosensory cortex. Both AL and FR animals were than randomly divided into four groups according to the duration of the recovery period, i.e. 2, 7, 14 and 28 days post-injury (dpi). Control animals from both AL and FR group were not subjected to TBI.

3.1 FR Increases The Level of p-GR in both AL and FR Animals

Since FR activates glucocorticoid pathway controlling the inflammatory response after the injury, the changes in the levels of GR (94kDa), and its transcriptionally active form – pGR, a 97kDa GR phosphorylated on Ser^{232} in rats (corresponding to human Ser^{211}), were evaluated first, by WB analysis (Fig. 1A and 1B, respectively). Two-way ANOVA of data revealed no significant effect of FR or time, as well as of their interaction for total GR levels (Fig. 1A). However, for the levels of p-GR (Ser^{232}), significant effects of FR ($F(1,40)=13.400$, $p<0.05$), time ($F(4,40)=16,279$, $p<0.05$) and

FR x time ($F(4,40)=5,603$, $p<0.05$) were observed. Namely, in AL group p-GR^{Ser232} was significantly increased in the 2nd, 7th, 14th and 28th day (61%, 63%, 126%, and 76%, respectively) post-injury (dpi) compared to AL control (Fig. 1B; *$p<0.05$). In FR group p-GR was even more significantly increased in the 2nd, 7th, 14th and 28th dpi (107%, 218%, 123%, and 97%, respectively) compared to FR control (Fig. 1B; \$$p<0.05$). Post hoc analysis showed a significant increase of p-GR (159%) on the 7th dpi in FR vs. AL rats (Fig.1B; #$p<0.05$). WB analysis did not reveal any significant differences in p-GR between AL and FR physiological controls (Fig.1B).

3.2. FR alters the Cellular translocation of p-GR

Given that p-GR (Ser$^{232)}$ levels were significantly increased in the ipsilateral cortex of both AL and FR animals following injury (Fig. 1B), we next analyzed the intracellular distribution of p-GR^{Ser232} in the cortical area surrounding the lesion. The representative images of Alexa Fluor-555 labelled p-GR and SYTOX® green labelled nucleic acids are shown in Fig.2. The comprehensive ImageJ analysis of colocalization revealed that the Pearson correlation coefficient (PCC) between Alexa Fluor-555 labelled p-GR and SYTOX® green labelled nucleic acids was indicative of a positive correlation (Table 3). Moderate positive correlation between p-GR^{Ser232} and SYTOX® green was found on 2nd, 7th and 14th dpi in AL animals, and on 2nd and 14th dpi in FR animals (Table 3). However, on 7th dpi in FR animals the strong positive correlation was observed in the vicinity of the lesion (PCC 0.748 ± 0.020). By the 28th dpi in both AL and FR animals PCC values corresponded to levels observed in the brains of control animals (Table 3).

Further ImageJ analysis of number of SYTOX Green- and p-GR^{Ser232}-labeled nuclei revealed that there is significant increase in number of SYTOX Green-labeled nuclei in

both AL and FR animals on 2nd, 7th and 14th dpi (Fig. 3). The most pronounced increase in number of SYTOX Green-labeled nuclei in AL animals occurs on 14th dpi, while in FR animals the highest elevation in number of SYTOX Green-labeled nuclei occurs on 2nd dpi (Fig. 3). However, the analysis of number of p-GR-labeled nuclei, and consequent correlation to the SYTOX Green data revealed that percentage of p-GR^{Ser232}-labeled nuclei in the overall number of SYTOX Green-labeled nuclei differs considerably among AL and FR animals (Fig. 3). In AL animals a significant decrease in percentage of p-GR^{Ser232} to SYTOX Green-labeled nuclei occurs in 2nd and 7th dpi (3.5, 5 and 1.5 fold, respectively) as compared to AL control (*p<0.05; **p<0.005; p<0.001***). However, in FR animals a significant increase in percentage of p-GR^{Ser232} to SYTOX Green-labeled nuclei can be observed in 2nd and 7th dpi (0.8 and 3.2 fold, respectively) as compared to FR control ($p<0.05; $p<0.005).

3.3. FR increases Sgk-1 mRNA Expression Levels

To demonstrate the GR transcriptional activity, we next examined the expression levels of the serum and glucocorticoid-regulated kinase 1 (Sgk-1) that is predominantly under acute transcriptional control by glucocorticoids. RT-PCR analysis revealed that although p-GR^{Ser232} levels were significantly increased in both AL and FR animals Sgk-1 gene expression was increased only in FR, but not in the AL group (Fig. 4). The most prominent increase in Sgk-1 gene expression in FR animals was detected on the 7th and 14th dpi (142% and 71 %, respectively). This increase corresponds to a significant elevation in p-GR in FR animals (Fig. 1B) as revealed by WB, and to strong positive correlation between p-GR and SYTOX® green especially on 7th dpi in FR animals (Fig. 2 and Table 3). Both WB- and IHC-obtained results showed the highest levels of p-GR^{Ser232} expression and highest p-GR nuclear localization on 7th

dpi, which corresponds to the highest levels of Sgk-1 gene expression, indicating that FR enhances p-GR^{Ser232} translocation to nucleus and its transcriptional activity following TBI. At the same time, the expression of Sgk-1 in AL animals was at the control level values throughout the recovery period, with a small but significant decrease in the 7th dpi compared to control (Fig. 4), which was not in correspondence with the increased p-GR levels revealed by WB (Fig.1B). The results for both Sgk-1 and p-GR in the FR group imply that p-GR achieves its effects in these animals using transcriptional signaling pathways.

3.4. FR decreases 11β-HSD1 Protein Expression Levels

Since 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) is the major regulator of the tissue-specific effects of circulating glucocorticoid excess, we next analyzed the protein level of this enzyme in the ipsilateral brain tissue (Fig. 5). Two-way ANOVA with FR and time as factors revealed a significant effect of FR ($F(1,40)=15.243$, $p<0.05$), time ($F(4,40)=13.140$, $p<0.05$) and FR x time ($F(4,40)=9.544$, $p<0.05$) on 11β-HSD1 levels. They were significantly increased in AL group on the 2nd, 7th, 14th and 28th dpi (24%, 43%, 39%, and 37% respectively) compared to AL control (*$p<0.05$). Oppositely, in the FR group, levels of 11β-HSD1 were significantly decreased on the 14th and 28th dpi (36% and 51%, respectively) compared to FR control (#$p<0.05$). Post hoc analysis showed a significant decrease of 11β-HSD1 on the 2nd, 7th, 14th and 28th dpi in FR rats (28, 54, 75 and 88%, respectively) vs. AL rats at matching time points ($p<0.05$). However, WB analysis did not reveal any significant differences in 11β-HSD1 between AL and FR physiological controls.

3.5. FR increases NFκB(p65) Protein Expression Levels

Considering that our previous results showed that FR acts neuroprotective[14,15], we next sought to evaluate changes in the level of NFκB(p65) protein, the transcriptional factor vital both for inflammation and neuronal survival. WB analysis was performed in the ipsilateral cortex of AL and FR rats following injury (Fig. 6). Two-way ANOVA with FR and time as factors revealed a significant effect of FR ($F(1,50)=16,605$, $p<0.05$), time ($F(4,50)=8,164$, $p<0.05$) and FR x time ($F(4,50)=6,774$, $p<0.05$). In AL group NFκB(p65) was significantly increased in the 2nd and 28th dpi (52% and 37%, respectively) compared to AL control (*$p<0.05$). In FR group NFκB(p65) was significantly increased in the 2nd, 7th, 14th and 28th dpi (50%, 71%, 48%, and 49%, respectively) compared to FR control ($p<0.05$). Post hoc analysis showed a significant increase of NFκB(p65) in the 7th dpi in FR rats (72%) vs. rats at matching time points in the AL group (#$p<0.05$). WB analysis did not reveal any significant difference in NFκB(p65) between AL and FR physiological controls.

3.6. FR increases HSP 70 Protein Expression Levels on the 7th dpi

The 70kDa heat shock protein (HSP70) protects cells against a variety of stress conditions and prevents apoptosis in different cell types[18]. WB analysis of HSP70 was performed in the ipsilateral cortex of AL and FR rats following injury (Fig. 7). Two-way ANOVA with FR and time as factors revealed a significant effect of FR ($F(1,50)=14,372$, $p<0.05$), time ($F(4,50)=8,217$, $p<0.05$) and FR x time ($F(4,50)=6,544$, $p<0.05$). In the AL group HSP70 stayed unchanged throughout the recovery period compared to AL control. In FR group HSP70 was significantly increased in the 7th dpi (35%) compared to FR control ($p<0.05$). Post hoc analysis showed a significant increase of HSP70 on the 7th dpi in FR (52%) vs. AL group (#$p<0.05$). No significant differences in HSP70 were observed between physiological AL and FR controls.

3.7. Correlation analysis of p-GR, NFkB and HSP70 Expression

The Pearson correlation coefficient (PCC) was chosen to assess associations between the expression of p-GR with the expression of transcription factor NFkB and chaperon HSP70 in cortex of animals fed AL or FR prior to TBI. Notably, p-GR expression was positively correlated with NFkB in cortical tissue of FR animals (r= 0.9473, R square= 0.8973, p= 0.0144) (Fig. 8B), with no correlation in this brain region in AL fed animals (Fig. 7A). However, no significant correlation between the expression of p-GR and HSP 70 protein was observed in the cortex in any of the experimental groups (Fig. 8C,D).

4. Discussion

In the present study, we extended our previous findings of the neuroprotective role of FR in the rat model of TBI[13,14,15] and examined the effects of FR on GR signaling and GC metabolism in the brain (11β-HSD1) as well as the expression of related neuroprotective molecules, NFκB, and HSP70. The major finding of this report is that FR enhances the level of transcriptionally active p-GR^{Ser232} in the ipsilateral cortex and promotes its translocation to the nucleus. Moreover, FR significantly changed the levels of 11β-HSD1, NFκB(p65) and HSP70 in the ipsilateral cortex. These data suggest that the protective effects of FR are mediated by synchronized regulation of different factors, ameliorating secondary injury at multiple levels.

Several lines of evidence suggest that neuro-inflammatory processes, as a consequence of TBI, promote neurodegeneration[19]. Microglial cells represent the major propagator of the secondary injury process. These cells, as the innate immune cells of the

CNS, react to virtually any insult but their prolonged activation can induce detrimental neurotoxic effects by releasing a variety of cytotoxic substances, including pro-inflammatory cytokine TNF-α[20]. Numerous studies have shown that CORT exerts anti-inflammatory activities, as reflected by diminishing microglial activation and proinflammatory cytokine production, and its beneficial effects are ascribed to this property[21]. Our previous studies showed that FR significantly elevates the serum CORT levels (up to 3 times in non-lesioned controls and up to 7 times after TBI)[13], simultaneously suppressing microglial activation and proinflammatory cytokine production following injury[14]. Since the anti-inflammatory effects of CORT are mediated, *inter alia*, by direct interaction of the glucocorticoid receptor with the transactivation domain of NFκB[22], translocation of p-GR to the nucleus is essential to the anti-inflammatory action of GR.

Results presented in this study clearly demonstrate that AL and FR feeding regimens differently affect translocation of p-GR^{Ser232} to nuclei after TBI. Namely, AL feeding prevents translocation of p-GR^{Ser232} to nucleus, while FR significantly elevates the rate of translocation of p-GR^{Ser232} to nucleus following TBI, within the tissue surrounding lesion site. Consequently, in AL animals, the expression of Sgk-1 mRNA level, the marker of p-GR^{Ser232} transcriptional activity, was at the control level values throughout the recovery period, which corresponded to low level of nuclear localization of p-GR^{Ser232}. However, in the FR group the elevated Sgk-1 mRNA levels, fully corresponded to observed increase in p-GR translocation to nucleus, which clearly implies that p-GR achieves its effects in FR animals using transcriptional signaling pathways. Thus, we hypothesize that CORT elevation in AL brains preferentially

mediates the non-transcriptional effects, while it seems that in FR brains, both transcriptional and non-transcriptional functions of glucocorticoid receptor are maintained. Elevated Sgk-1 mRNA level, observed only in brains of FR animals, together with elevated translocation of p-GR^{Ser232} to the nuclei following injury supports the hypothesis.

Prolonged and elevated CORT levels are commonly linked to neurodegeneration[11]. High levels of CORT and p-GR^{Ser232}, as well as Sgk-1 mRNA, coincide in time with the increase in synaptic plasticity proteins and anti-apoptotic proteins observed on the 7th dpi in FR animal[13,15]. Thus, FR may activate different pathways near the injury site that protect neurons from inflammation-induced neuronal cell death, but also from elevated levels of CORT itself.

Traumatic injury to the brain and spinal cord results in increased levels of NFkB activity in cells within and surrounding the site of injury including neurons, astrocytes, and microglia[23]. The NFkB activity can remain elevated for long periods (weeks to months) following TBI and likely contribute to associated inflammatory processes[23]. Since the present study revealed that FR significantly elevates NFκB(p65) levels in the ipsilateral cortex throughout the recovery period, a strong inflammatory response in the brains of FR animals could be expected. However, our previous study revealed that FR restraint inflammation by suppressing microglial activation and TNFα over-expression following TBI[14]. That is why the cell-specific action of NFκB should be taken into account, since activation of NFκB specifically in neurons protects them against degeneration, whereas activation of NFκB in microglia triggers the expression of cytokines that promote inflammation and neuronal degeneration[23]. Namely, cell culture

studies have clearly shown that activation of NFκB in neurons protects them against excitotoxic and metabolic insults relevant to the pathogenesis of stroke, including glucose deprivation and exposure to glutamate[23]. Results of the present study indicate that FR, may act through 'switching' the NFκB activation from microglial cells to neurons, thus favoring neuroprotection in the brains of FR animals. Indeed, the same level of NFκB(p65) expression (2nd dpi) in both experimental groups of animals results in a drastically different outcome - inflammation and neuronal cell death in AL, and absence of both inflammation and neurodegeneration in FR animals[14]. Thus, we presume that FR-promoted GR actions are most likely cell-specific and that in microglial cells GR binds to NFκB, disabling its transcriptional activity, hindering the proinflammatory cytokine transcription, while in neuronal cells GR does not interfere with NFκB activation, which leads to the transcription of neuroprotective factors. Moreover, an analysis of the correlation between p-GR and NFkB in FR animals revealed that p-GR was positively associated with NFkB(p65). Nevertheless, further analyses are needed to decipher the cell-specific FR-promoted GR actions in response to TBI.

It is also well documented that both GR and NFκB, exhibit direct anti-apoptotic effect by modulating the key regulators of the intrinsic apoptotic pathway, survival genes Bcl-2 and Bcl-xL, in a cell-specific manner[24]. Most pertinently, glucocorticoids increase the intracellular level of anti-apoptotic protein Bcl-2[25], while activated NFκB(p65 subunit) exerts its neuroprotective function via upregulation of anti-apoptotic Bcl-xL protein[24]. Our previous data showed that FR suppressed neuroapoptosis and promoted significant upregulation of antiapoptotic Bcl-2 and Bcl-xl mRNAs in the ipsilateral cortex following injury[15]. The upregulation of these anti-apoptotic genes in FR brains on 7th dpi

corresponds to the upregulation of p-GR^{Ser232} and NFκB(p65) levels, indicating the strong involvement and interplay of both transcriptional factors in the processes of FR-induced neuroprotection.

In models of brain injury, FR also resulted in a widespread heat shock protein 70 induction, particularly within cortical and hippocampal neuron[26]. Numerous studies using strategies to increase or inhibit HSP70 have shown that this chaperone protects the brain in experimental cerebral ischemia, neurodegenerative disease models, epilepsy, and trauma [27]. HSP70 protein is also involved in mediating cytoprotection via anti-apoptotic effects in a variety of cell types[18]. The present study revealed that the significant elevation of the HSP70 protein level on 7th dpi corresponds to the increase of p-GR^{Ser232} and NFκB(p65). Moreover, this HSP70 elevation coincided with the increase in anti-apoptotic proteins as previously described[15].

It has been observed that chronically applied synthetic GCs do not exhibit a protective effect that can be detected with elevated endogenous CORT concentrations in FR animals. This suggests that stress-induced by FR differs from other stressors or glucocorticoid therapy[28]. Namely, unlike synthetic, the effects of natural GCs are mediated by CORT protein carriers and 11β-HSD which, acting as a buffer system, protect the tissue from extremely high concentrations of these hormones [27,28]. Our results showed that 3-month-long FR significantly reduced the level of 11β-HSD1 in the ipsilateral cortex, which is consistent with the data showing that stress alone regulates the expression of 11β-HSD1 in a time- and tissue-dependent manner[29]. While short-term stressors or glucocorticoid therapy (2-10 days) lead to elevated 11β-HSD1 expression in the hippocampus[30] long-term stressors (1 month) lead to reduced expression of this

enzyme[29]. These data indicate that the increase in 11β-HSD1 activity in response to acute stress is a compensatory mechanism that enhances the negative feedback signal to "switch-off" the HPA axis. On the other hand, a decrease in the activity of 11β-HSD1 in chronic stress models is a protective response of an organism based on alleviating the harmful effects of excessive glucocorticoids in the tissue[31]. Overall, data indicate that the reduction in the activity of 11β-HSD1 has positive effects on the organism of animals.

It is also proposed that ghrelin, a “gut-brain” hormone, can act as a neuroprotective agent in experimental models of TBI and cerebral ischemia[32]. Proposed mechanisms include antiinflammation and antiapoptosis, through downregulating brain levels of TNF-α and IL-6 and reducing cortical levels of cleaved PARP-1 after TBI [33]. It has also been shown that ghrelin attenuates hippocampal neuronal damage by decreasing ratio of Bcl-2/Bax and inhibiting caspase-3 activation in a rat model of pilocarpine-induced seizures[34]. Since FR represents a pleiotropic approach it exerts its beneficial effects not just through different molecular cascades and transcription factors such as p-GR or NFκB within the single cell, but also through different levels of control such as the endocrine system of the body. Thus, it might be that FR acting through multiple levels synchronizes the entire organism to respond to some harmful stimulus in the best way possible, reminding us constantly that FR is much more than just the sum of its parts.

Although the paradigm of pre-injury caloric restriction may appear as a treatment with narrow clinical relevance, data from the animal studies showed that FR is more efficient in improving functional recovery if applied pre- than post-injury[35]. Moreover, decreased caloric intake after the injury is associated with the increased mortality rates of TBI patients in clinical settings due to a triggered cellular energy crisis as a hallmark of

post-TBI early stages[36]. In such conditions, the introduction of FR as another stressor can even further deteriorate TBI-induced energy crisis.

Since the effects of FR are considered to result from hormetic mechanisms, preventive life-style changes could lessen the severity of poor outcomes in the event of TBI. As for clinical relevance, our data suggest that such a regimen could have beneficial effects if implemented prior to any surgical interventions that put the CNS at risk. However, the safe therapeutic implementation of such restrictive feeding in humans needs additional adjustments.

5. Conclusions

In conclusion, our data provide evidence that FR introduced before injury strongly affects GR signaling after the injury, by significant elevation of p-GR levels, its translocation to the nucleus and subsequent enhancement of its transcriptional activity. Moreover, other protective factors such as 11β-HSD1, NFκB(p65) and HSP70 act together with GR, adding to it's anti-inflammatory and neuroprotective effects. This study gives further insights into the molecular networks underlying FR protective effects following traumatic brain injury.

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Table 1. Pelletized commercial diet content.

Content	Percentage
Protein	20 min. %
Carbohydrate	59.9 %
Fat	3.7 %
PUFA/SFA	1.3
n-3/n-6 PUFA	0.05
fiber	5.6 %
ash	7.6 %
Phosphorus	0.5 min. %
Phosphorus usable	0.4 % min.
Sodium	0.15-0.25 %
Lysine	0.9 % min.
Met + Cis	0.75 % min.
amount of vitamins and minerals per 1kg of standard rat food	
Vit. A	10000 IJ
Vit. D	1600 IJ
Vit. E	25 mg
Vit. B12	0.02 mg
Fe	100 mg
Cu	20 mg
Zn	100 mg
Mn	30 mg
J	0.5 mg
Se	0.1 mg

SFA, Saturated fatty acids; PUFA, polyunsaturated fatty acids; n-3, omega-3; n-6, omega-6

Schematic representation 1. Schematic presentation of the experimental procedure

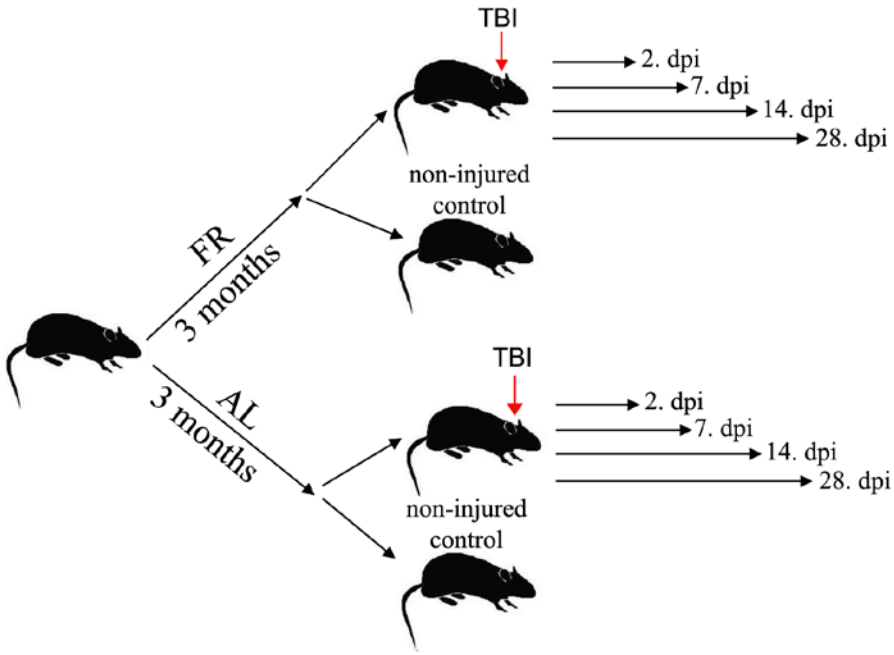

Table 2. Primary antibodies used for Western blot analysis.

Antibody against	Catalogue No.	Host/clonality	Manufacturer	Dilution
GR	sc-1004	rabbit polyclonal	Santa Cruz	1:750 in 5% milk
p-GR (Ser^{211})	4161	rabbit polyclonal	Cell Signaling	1:500 in 3% BSA
11β-HSD1	AF3397	goat polyclonal	R&D Systems	1:1000 in TBST
NFκB(p65)	sc-372	rabbit polyclonal	Santa Cruz	1:1500 in 5% milk
HSP70	sc-1060	goat polyclonal	Santa Cruz	1:1000 in TBST

Table 3. Colocalisation between p-GR and nucleic acids. Data shown are means ± s.e.m. p values are from a two-tailed t-test (ns p>0.05; *p<0.05; **p<0.01; ***p<0.001).

Time point / Feeding	control	2 dpi	7 dpi	14 dpi	28 dpi
AL	**0.332 ± 0.021**	** **0.499 ± 0.017**	** **0.502 ± 0.022**	** **0.589 ± 0.033**	n.s. **0.369 ± 0.020**
FR	**0.352 ± 0.026**	** **0.584 ± 0.037**	*** **0.748 ± 0.020**	* **0.536 ± 0.051**	n.s. **0.318 ± 0.023**

Figure legends

Figure 1. The protein levels of GR and p-GR (Ser^{232} phosphoisoform) in the ipsilateral cortex of AL and FR animals following cortical injury, as revealed by WB. **A)** Protein levels of GR, accompanied by a representative immunoblot; **B)** Protein levels of p-GR(Ser^{232}), accompanied by a representative immunoblot; The data represent mean±S.E.M.;*p<0.05 vs. AL control; $p<0.05 vs. FR control; #p<0.05 FR vs. AL.

Figure 2. Merged images of p-GR (red)/SYTOX green (blue) immunostaining in AL and FR animals after TBI. Lane (A) and (B), representative images of p-GR/SYTOX green immunostaining of the AL group. Lane (C) and (D), representative images of p-GR/SYTOX green immunostaining of the FR group. Lanes (B) and (C), colocalized pixel map where every colocalized pixel is represented as a white dot within the merged image. The numbers in the bottom right corner in the images in lanes (B) and (C) represent the mean PCC value for every time point of both AL and FR experimental groups. Magnification 20x/1.3. Scale bar: 50μm.

Figure 3. Representation of quantification of p-GR^{Ser232} translocation to nuclei. Number of SYTOX Green-labeled and p-GR^{Ser232} – labeled nuclei were quantified around the site of the lesion. Entire bar represents the total number of nuclei (SYTOX Green-labeled nuclei), light-gray part of the bar represents the p-GR-labeled nuclei within the total number of nuclei quantified. The percents abowe the bars represent the percentage of p-

GR-labeled nuclei in the total number of nuclei. *p<0.05; *p<0.005; *p<0.001 *vs*. AL control; $p<0.05; $p<0.005; $p<0.001 *vs*. FR control.

Figure 4. Levels of Sgk-1 mRNA in the ipsilateral cortex of AL and FR animals following cortical injury, as revealed by Real-time PCR. *p<0.05 *vs*. AL control; $p<0.05 *vs*. FR control; #p<0.05 FR *vs*. AL.

Figure 5. Protein levels of 11β-HSD1 in the ipsilateral cortex of AL and FR animals following cortical injury, as revealed by WB. The graph is accompanied by a representative immunoblot. The data represent mean±S.E.M.; *p<0.05 *vs*. AL control; $p<0.05 *vs*. FR control; #p<0.05 FR *vs*. AL.

Figure 6. Protein levels of NFκB(p65) in the ipsilateral cortex of AL and FR animals following cortical injury, as revealed by WB. The graph is accompanied by a representative immunoblot. The data represent mean±S.E.M.; *p<0.05 vs. AL control; $p<0.05 vs. FR control; #p<0.05 FR vs. AL.

Figure 7. Protein levels of HSP70 in the ipsilateral cortex of AL and FR animals following cortical injury, as revealed by WB. The graph is accompanied by a representative immunoblot. The data represent mean±S.E.M.; $p<0.05 vs. FR control; #p<0.05 FR vs. AL.

Figure 8. Correlation analysis of p-GR, NFkB(p65) and HSP70 in AL and FR rat cortex after traumatic brain injury. The expression profile of p-GR is closely correlated with NFkB(p65) after TBI in FR animals (r= 0.9473, R square= 0.8973, p= 0.0144), (B) but not in cortex of AL animals (A); There were no correlations between expression of p-GR and HSP70 in AL or FR experimental group (C,D).

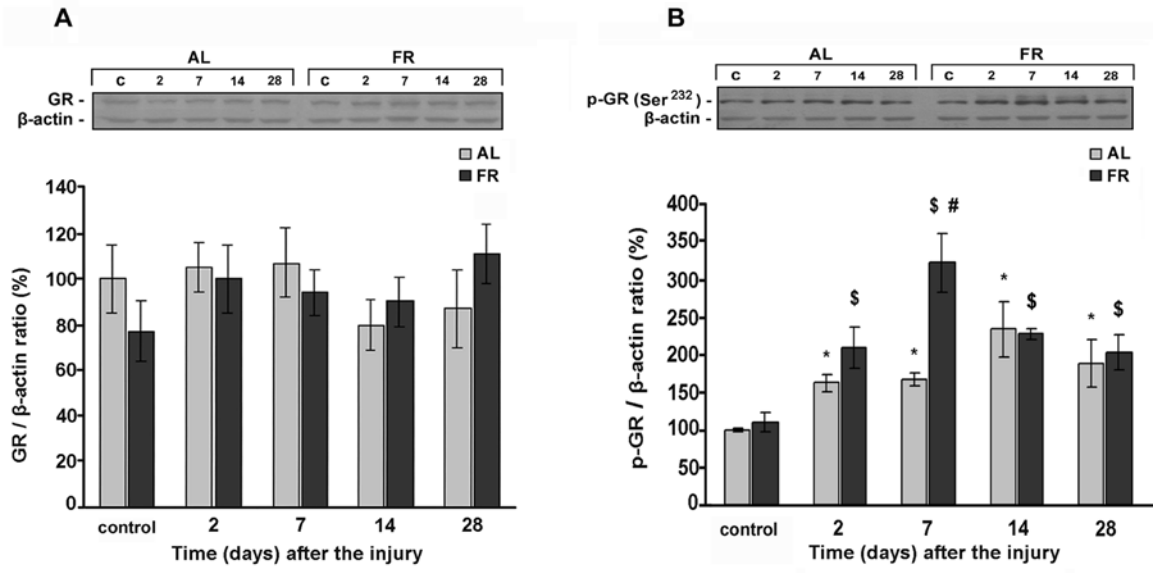
A
AL
FR
C 2 7 14 28 C 2 7 14 28
GR -
β-actin -
AL
FR
GR / β-actin ratio (%)
0 20 40 60 80 100 120 140
control 2 7 14 28
Time (days) after the injury
B
AL
FR
C 2 7 14 28 C 2 7 14 28
p-GR (Ser 232) -
β-actin -
AL
FR
p-GR / β-actin ratio (%)
0 50 100 150 200 250 300 350 400
$ #
$
$
$
*
*
*
control 2 7 14 28
Time (days) after the injury

Fig. 1.

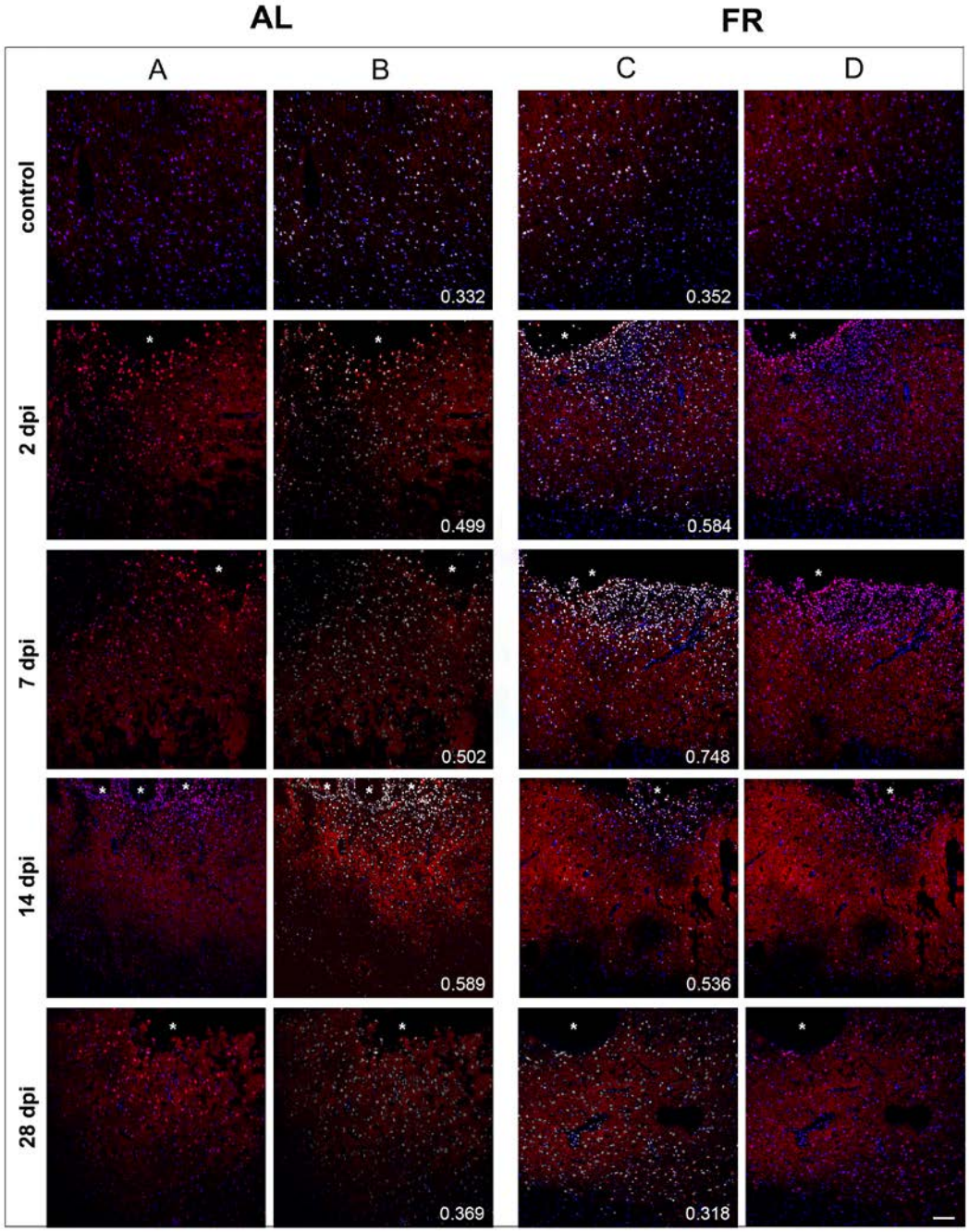
AL
FR
A
B
C
D
control
2 dpi
7 dpi
14 dpi
28 dpi
0.332
0.352
0.499
0.584
0.502
0.748
0.589
0.536
0.369
0.318

Fig. 2.

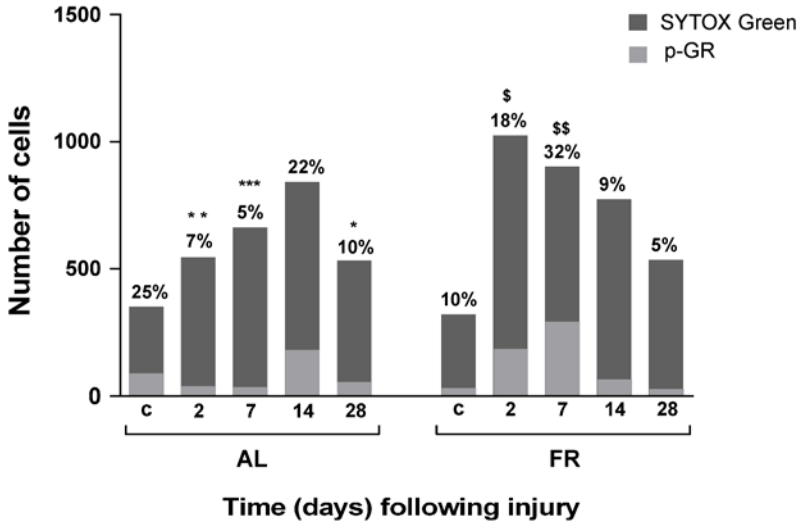
SYTOX Green
p-GR
Number of cells
1500
1000
500
0
25%
* *
7%
***
5%
22%
*
10%
10%
$
18%
$$
32%
9%
5%
c
2
7
14
28
c
2
7
14
28
AL
FR
Time (days) following injury

Fig. 3.

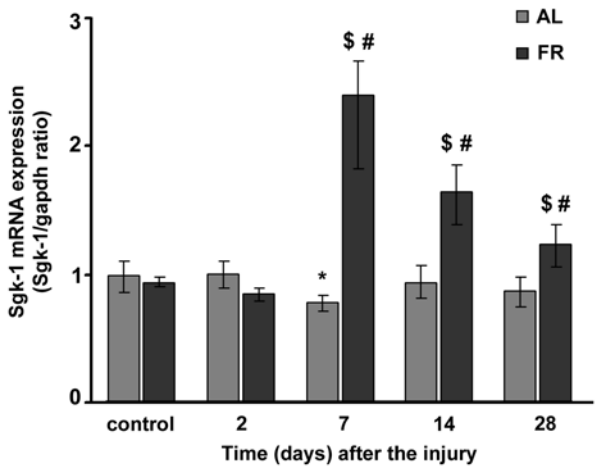
AL
FR
Sgk-1 mRNA expression
(Sgk-1/gapdh ratio)
3
2
1
0
$ #
$ #
$ #
*
control
2
7
14
28
Time (days) after the injury

Fig. 4.

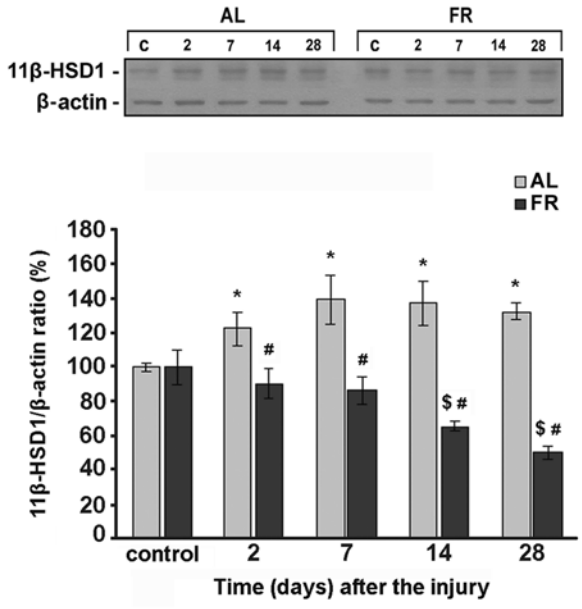
AL
FR
C
2
7
14
28
C
2
7
14
28
11β-HSD1 -
β-actin -
AL
FR
11β-HSD1/β-actin ratio (%)
180
160
140
120
100
80
60
40
20
0
*
#
$ #
control
2
7
14
28
Time (days) after the injury

Fig. 5.

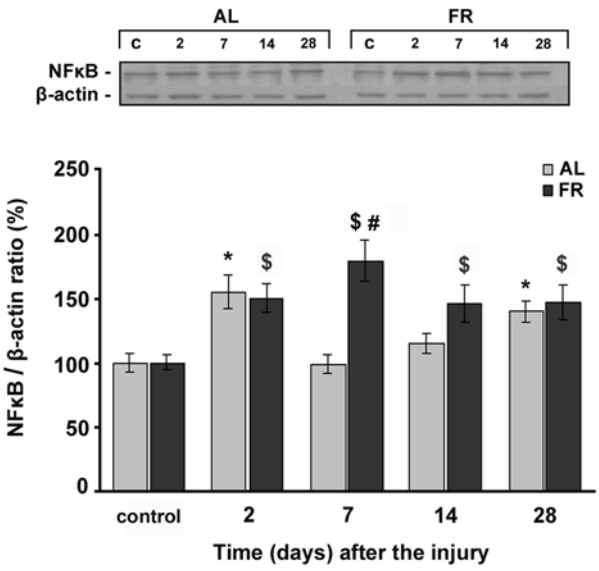
AL
FR
C
2
7
14
28
C
2
7
14
28
NFκB -
β-actin -
250
200
150
100
50
0
NFκB / β-actin ratio (%)
AL
FR
*
$
$ #
$
*
$
control
2
7
14
28
Time (days) after the injury

Fig. 6.

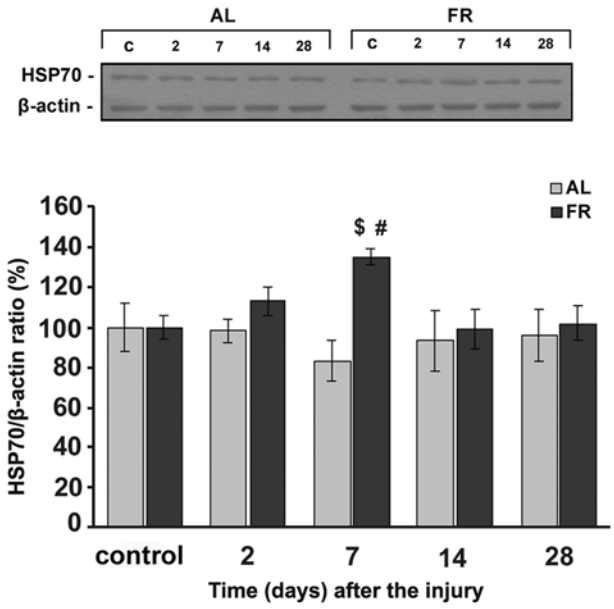
AL
FR
C
2
7
14
28
C
2
7
14
28
HSP70 -
β-actin -
AL
FR
$ #
HSP70/β-actin ratio (%)
160
140
120
100
80
60
40
20
0
control
2
7
14
28
Time (days) after the injury

Fig. 7.

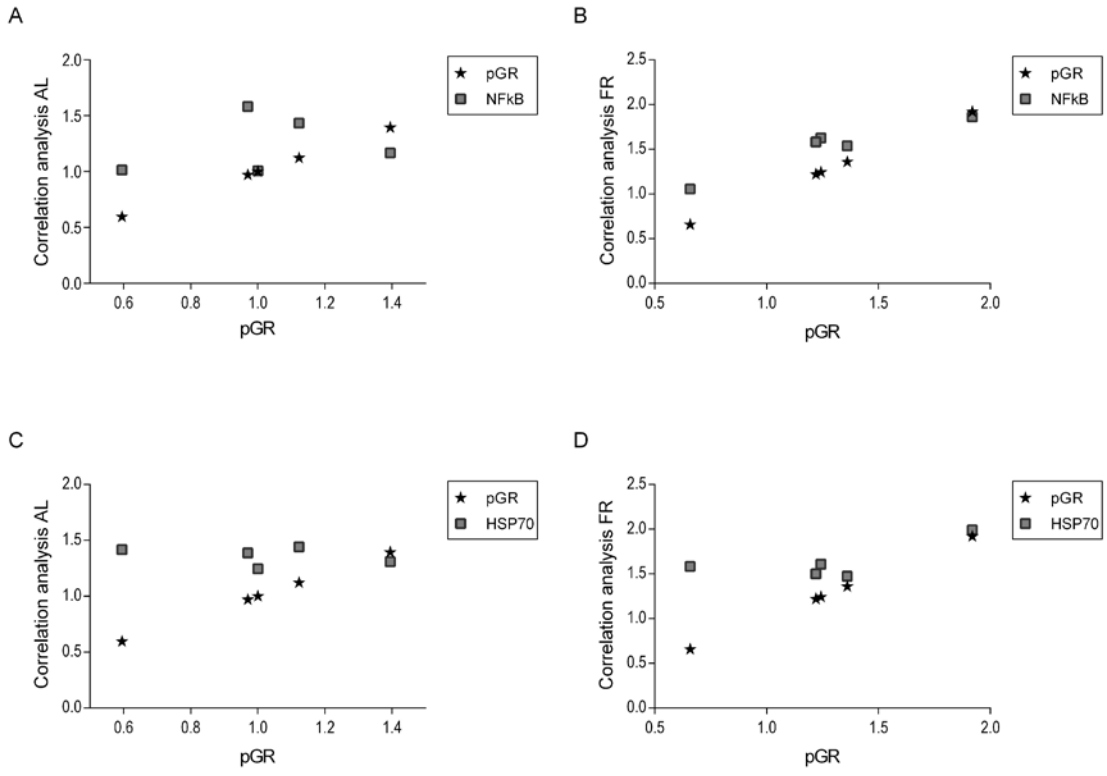
A
Correlation analysis AL
pGR
NFkB
B
Correlation analysis FR
pGR
NFkB
C
Correlation analysis AL
pGR
HSP70
D
Correlation analysis FR
pGR
HSP70

Fig. 8.