Cerebrospinal fluid and serum chemokines concentrations in differentiation CNS brain tumors patients from non tumoral individuals.

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**Key words:** biomarkers; CNS brain tumors; CCL-2; IL-8; sICAM-1

**Abstract**

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**Introduction**

Gliomas are ranked as the most aggressive brain tumors; they present high expression of invasion mediators such as angiogenic factors and chemokines [1,2]. Chemokines are small in size proteins, which are produced and released locally to be destined for local action [3].

Monocyte chemotactic protein-1 (MCP-1/CCL2) has been found in different tumor samples and may be recognize as a potential regulator of cancer progression [4,5]. *In vivo* studies showed that a significant source of CCL2 is tumor epithelium [5]. Additionally, tumor cells themselves may be major source of this protein. Expression and localization of mRNA and protein for CCL2 in human malignant glioma was found by *Takeshima et al.* [4]. Studies of other authors revealed, that antibody-mediated blockade of CCL2 had an influence on the survival prolongation on mice or human glioma cells [6]. These findings suggest that CCL2 may be a potential therapeutic target.

Interleukin-8 (IL-8), also known as CXCL8, is a member of the CXC family of chemokines [7]. Similarly, to the CCL2, IL-8 may be produced by the tumor cells itself and/or released by immune cells activated in response to tumor cells growth [8]. During inflammation IL-8 takes part in carcinogenesis by acting directly on epithelial cells via NFKB1 pathway signaling. Additionally, IL-8 expression by tumor cells stimulates cancer cells proliferation, migration, as well as invasion [8,9].

Intercellular adhesion molecule 1 (ICAM-1/CD54) is a nearly ubiquitous transmembrane glycoprotein. Elevated levels of this protein were related with certain malignancies [10]. Soluble ICAM-1 (sICAM-1) has been recognized as an indicator of vascular endothelial cell activation or damage [11]. It also has an inhibiting role for transmembrane ICAM-1 mediated activities such as sensitivity of tumor cells to NK cell-mediated lysis [12].

A diagnostic quantitative tests (biological markers) for brain tumors based on biochemical analysis of blood and/or CSF are still lacking, therefore the aim of the current study was the evaluation of serum and CSF concentrations of CCL2, IL-8, and ICAM-1 in patients with neuroepithelial tissue tumors as compared to patients with unruptured intracranial aneurysms (UIAs) with no history of cancer as a comparative group. UIA usually is asymptomatic and only discovered incidentally [13]. It would be of great interest to establish characteristic biomarkers pattern measurable in biological fluids distinguishing patients with CNS tumors from non tumoral subjects. Moreover, our studies may indicate potential factors, which may indicate the directions for targeted therapy.

**Materials and methods**

*Subjects*

The study group consisted of 20 patients (11 males/9 females, mean age 56 years, range 39-73 years) with previously untreated cerebral tumors of neuroepithelial tissue, which originated from astrocytic tumors (**Table 1**). The comparative group was composed of 20 non tumoral subjects (4 males/16 females, mean age 56 years, range 30-70 years) with unruptured intracranial aneurysms (UIAs), with no history of cancer. The study was conducted in agreement with the Helsinki-II-declaration and was approved by the Bioethics Human Research Committee of the Medical University of Bialystok. All subjects included to the study gave their written informed consent.

**Table 1.** Histopathological examination results and WHO grading of particular CNS tumors subjects included to the study.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **No.** | **Histopathological**  **examination** | **WHO** | **GFAP** | **p53** | **Ki-67** | **EGFR** | **IDH1** |
| **1** | **Diffuse astrocytoma** | **2** | **(+)** | **(+)** | **(+)**  **in about 2% cells** | **(-)** | **(-)** |
| **2** | **Glioblastoma** | **4** | **(+)** | **(+)**  **in about 10% cells** | **(+)**  **in about 30% cells** | **(++)** | **(-)** |
| **3** | **Glioblastoma** | **4** | **(+)** | **(+)**  **in about 20% cells** | **(+)**  **in about 30% cells** | **(++)** | **(-)** |
| **4** | **Anaplastic astrocytoma** | **4** | **(+)** | **(+)**  **in the single cells** | **(+)**  **in about 20% cells** | **(+)** | **(-)** |
| **5** | **Diffuse astrocytoma** | **2** | **(+)** | **(+)**  **in the single cells** | **(+)**  **in about 1% cells** | **(-)** | **(-)** |
| **6** | **Glioblastoma** | **4** | **(+)** | **(+)** | **(+)**  **in abaut 60% cells** | **(+)**  **in parts of**  **neoplastic tissue** | **(+)** |
| **7** | **Glioblastoma** | **4** | **(+)** | **(+)** | **(+)**  **in about 20% cells** | **(+)** | **(+)** |
| **8** | **Glioblastoma** | **4** | **(+)** | **(+)** | **(+)**  **in about 40% cells** | **(+)** | **(+)** |
| **9** | **Gliosarcoma** | **4** | **(+)** | **(+)** | **(+)**  **in about 30%** | **(++)** | **(+)** |
| **10** | **Glioblastoma** | **4** | **(+)** | **(+)** | **(+)**  **in about 30% cells** | **(+)** | **(+) focally** |
| **11** | **Anaplastic glioma** | **3** | **(+)** | **(+)** | **(+)**  **in about 20%** | **(+)** | **(-)** |
| **12** | **Glioblastoma** | **4** | **(+)** | **(+)**  **in about 30% cells** | **(+)**  **in about 20% cells** | **(+)** | **(-)** |
| **13** | **Glioblastoma** | **4** | **(+)** | **(+)** | **(+)**  **in about 40% cells** | **(+)** | **(+)**  **in the single cells** |
| **14** | **Glioblastoma** | **4** | **(+)** | **(+)**  **in about 30% cells** | **(+)**  **in about 80% cells** | **(-)** | **(-)** |
| **15** | **Pilocytic astrocytoma** | **1** | **(+)** | **(+)**  **in single cells** | **(+)**  **in 2%** | **(-)** | **(-)** |
| **16** | **Glioma** | **4** | **(+)** | **(+)**  **in 40% cells** | (+) in 20% cells | **(+)** | **(-)** |
| **17** | **Glioblastoma** | **4** | **(+)** | **(+)**  **in 20 cells** | **(+)**  **in 30% cells** | **(++)** | **(-)** |
| **18** | **Glioblastoma** | **4** | **(+)** | **(+)**  **in about 10% cells** | **(+)**  **in about 10% cells** | **(+)** | **(+)** |
| **19** | **Glioblastoma** | **4** | **(+)** | **(+)**  **in about 10% cells** | **(+)**  **in about 10% cells** | **(+)** | **(-)** |
| **20** | **Anaplastic astrocytoma** | **3** | **(+)** | **(+)** | **(+)**  **in about 10-15% cells** | **(+)** | **(-)** |

WHO – World Health Organization, GFAP – glial fibrillary acidic protein, p53 – tumor protein p53, Ki-67 – Ki-67 protein, EGFR – epidermal growth factor receptor, IDH1 – isocitrate dehydrogenase 1

*Sample collection and storage*

Procedures on patients were performed under a general anesthetic during the neurosurgery at the Department of Neurosurgery of the Clinical Medical Hospital in Bialystok. Craniotomy has been performed on all subjects included in the study. In patients undergoing tumor resection its size and localization was tailored according to location and size of tumor, preferred access route and surrounding anatomy. Clipping of an aneurysm required opening in fronto-temporal region (aka pterional craniotomy). Regardless of operation type or location, after placing patient’s head in a three-pin Mayfield headholder the surgical field has been prepared in standard fashion. Skin incision preceded lifting of bone flap and lancing of dura mater, which allowed to visualize arachnoid membrane and subarachnoid space. With the aid of operating microscope subarachnoid space has been carefully opened and CSF aspirated with single-use, sterile syringe and soft venous catheter. Aforementioned steps were taken in the very beginning of each procedure, before any bleeding may occur. This routine allowed to keep CSF clean of blood and not mixed with warm saline solution used as an irrigation.

All patients’ blood samples were drawn without stasis. Tubes with blood collected without anticoagulant and CSF samples were centrifuged for 20 minutes at 1000 x g. Obtained serum and CSF supernatant were stored at -80 0C until further analysis.

***CSF and serum CCL2, IL-8, and ICAM-1 concentrations evaluation***

Concentrations of CCL2 were measured using ELISA Quantikine® Human CCL2/CCL2 Immunoassay kit (Catalogue number: DCP00; R&D Systems Europe Ltd., Abingdon, England) according the manufacturer’s instruction. Samples were diluted 2-fold prior analysis. The manufacturer of assay kit referred to the intra-assay coefficient of variation (CV%) as 7.8% at CCL2 mean concentration of 76.7 pg/mL, SD = 6.0 pg/mL.

Concentrations of IL-8 were measured using ELISA Quantikine® Human CXCL-8/IL-8 Immunoassay kit (Catalogue number: D8000C; R&D Systems Europe Ltd., Abingdon, England) according the manufacturer’s instruction. Samples were not diluted prior analysis. The manufacturer of assay kit referred to the intra-assay coefficient of variation (CV%) as 5.6% at IL-8 mean concentration of 168 pg/mL, SD = 9.4 pg/mL.

Concentrations of sICAM-1 were measured using ELISA Quantikine® Human ICAM-1/CD54 Allele-specific Immunoassay kit (Catalogue number: DCD540; R&D Systems Europe Ltd., Abingdon, England). According to the ELISA protocol CSF and serum were diluted 20-fold prior analysis. The manufacturer of assay kit referred to the intra-assay coefficient of variation (CV%) as 3.7% at ICAM-1 mean concentration of 4.61 ng/mL, SD = 0.17 ng/mL.

The obtained results were statistically analyzed with the use of the STATISTICA 12.0 PL software (StatSoft Inc., Tulsa, USA). The concentrations of parameters tested did not follow a normal distribution in the preliminary statistical analysis (X2-test), thus nonparametric statistical analysis was employed. The Mann-Whitney test was used in order to compare two independent samples. Correlation coefficients were obtained by applying Spearman’s rank method. If not stated otherwise, the values for each given measured variable are presented as medians and interquartile ranges. Differences were considered statistically significant for P<0.05. Receiver operator characteristic (ROC) curves were generated to calculate the areas under the ROC curves (AUCs). The Youden index, a function of sensitivity and specificity, was estimated to indicate an optimal threshold value (cut-off point) for parameters tested. Youden index is a commonly used measure to the evaluation of the effectiveness of new biomarkers and gives equal weight to sensitivity and specificity for the values of the biomarker tested [14].

**Results**

Regardless of the parameter tested and the patients group (CNS brain tumors or non tumoral UIAs), statistically relevant differences were found between concentrations obtained in CSF compared to values obtained in serum for all proteins tested (P<0.05). Significant differences were found between the study groups in CSF for IL-8, while in serum differences were obtained for CCL2 and sICAM-1 (**Table 2**). Correlation coefficient analysis did not reveal relation between concentrations of proteins tested in the serum with values in the CSF, except moderate correlations for IL-8 in CNS brain tumors (R=0.50, P=0.024) and for CCL2 in UIAs (R=0.45, P=0.044).

**Table 2**. Serum, CSF, and Indexes values obtained in patients with CNS brain tumors as compared to non tumoral individuals with UIAs. Values are present as median and interquartile range.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **CNS brain tumors** | **UIAs** | **P-value** |
| **Serum** | | | |
| **CXCL8/IL-8** | 13.01 (6.85-16.45) | 9.98 (9.02-12.71) | NS |
| **CCL2/MCP-1** | 151.84 (123.89-213.70) | 247.40 (217.10-351.40) | **0.002** |
| **ICAM-1/CD54** | 137.48 (118.70-190.04) | 182.69 (142.39-232.10) | **0.026** |
| **CSF** | | | |
| **CXCL8/IL-8** | 65.13 (45.36-103.00) | 30.48 (23.81-36.82) | **0.000** |
| **CCL2/MCP-1** | 409.70 (207.34-735.30) | 458.70 (366.00-726.00) | NS |
| **ICAM-1/CD54** | 16.68 (6.78-25.84) | 19.22 (13.90-23.76) | NS**\*** |
| **Indexes** | | | |
| **CXCL8/IL-8** | 7.72 (3.91-9.81) | 2.90 (2.13-4.19) | **0.002** |
| **CCL2/MCP-1** | 2.74 (1.39-4.74) | 1.71 (1.42-2.03) | NS |
| **ICAM-1/CD54** | 0.12 (0.04-0.24)**A** | 0.11(0.06-0.15)**B** | NS**\*** |

\* P-value was calculated based on the obtained values for sICAM-1 in the CSF (only 6 concentrations for the CNS brain tumors and only 3 concentrations for the UIAs), so the interpretation is limited

**A** sICAM-1Indexes were calculated only for 6 patients, **B** sICAM-1Indexes were calculated only for 3 patients

***Serum and CSF results***

Serum IL-8 concentrations revealed a tendency to be higher in patients with brain tumors as compared to non tumoral UIAs, but obtained differences were not significant. CSF IL-8 concentrations were statistically elevated in CNS tumors patients as compared to brain aneurysm individuals (P=0.00). Serum CCL2 and sICAM-1 concentrations were significantly decreased in CNS tumors as compared to UIAs subjects (P=0.002 and P=0.026, respectively). CSF CCL2 and sICAM-1 concentrations revealed a tendency to be lower in CNS tumors as compared to UIAs subjects, but obtained differences were not significant. It should be highlighted, that sICAM-1 concentrations in brain tumors group were detectable only in 6 from 20 CSF samples. In the group of subjects with brain aneurysm sICAM-1 concentrations were detected only in 3 from 20 CSF samples (**Table 2**).

***Indexes results***

To exclude possible impairment of the blood-CSF barrier and/or blood brain barrier (BBB) functions as potential sources influencing concentrations of proteins tested, the CSF concentrations were related to the concentrations obtained in the serum by calculating the indexes, as it was described elsewhere [15]. IL-8Index was significantly higher in CNS brain tumors individuals as compared to UIAs group (P=0.02). We did not find the utility for the calculation neither for the CCL2Index, nor for sICAM-1Index (**Table 2**).

**Diagnostic criteria for proteins tested**

Among proteins tested in the serum higher area under the ROC curve (AUC) revealed CCL2 compared to ICAM-1 in differentiating subjects with CNS brain tumors from non tumoral subjects with UIAs. Both AUCs were statistically higher than AUC=0.5, which indicate their diagnostic usefulness (**Figure 1, Table 3**). AUC for CSF IL-8 was higher than for its index; however also both AUCs were statistically higher than AUC=0.5, which indicate their diagnostic usefulness for differentiating patients with primary brain tumors from non tumoral subjects with UIAs (**Figure 2, Table 3**).

**Table 3.** Diagnostic usefulness of serum CCL2, sICAM-1 and CSF IL-8 as well as IL-8Index.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Cut-off** | **Youden**  **Index** | **AUC** | **SE** | **P** | **Sensitivity [%]** | **Specificity [%]** | **PPV**  **[%]** | **NPV**  **[%]** | **ACC**  **[%]** |
| **Serum CCL2** | **216.20** | **0.65** | **0.793** | **0.080** | **0.000** | **85** | **80** | **81** | **84** | **83** |
| **Serum sICAM-1** | **138.68** | **0.40** | **0.708** | **0.083** | **0.012** | **55** | **85** | **79** | **65** | **70** |
| **CSF IL-8** | **39.48** | **0.60** | **0.860** | **0.062** | **0.000** | **80** | **80** | **80** | **80** | **80** |
| **IL-8Index** | **7.48** | **0.55** | **0.793** | **0.074** | **0.000** | **55** | **100** | **100** | **69** | **78** |

Cut-off – optimal cut-off based on the highest Youden index, AUC – area under the ROC curve, SE – Standard Error, PPV – positive predictive value, NPV – negative predictive value, ACC – diagnostic accuracy

**Discussion**

According to the literature free chemokines concentrations in normal human serum are mostly below the lower limit of detection. An explanation of this may be the presence of immune complexes in which the epitope is embedded [16].

However, our study revealed detectable concentrations, above the lower limit of detection, for IL-8 and CCL2 in all patients’ samples, regardless the material (serum/CSF) or study group (CNS brain tumors/non tumoral UIAs) analyzed (Figure 3).

Also serum sICAM-1 concentrations were detected in all samples tested, which was previously confirmed by the studies of *Nano et al*., as they revealed that sICAM-1 serum levels were not significantly increased in GBL and astrocytoma patients compared with another type of tumors (lung and kidney cancer) [10]. Surprisingly, CSF levels for adhesion molecule sICAM-1 were below the lower limit of the test sensitivity in most of the samples analyzed (we found sICAM-1 concentrations only in 6 of the 20 CSF samples of the CNS brain tumors patients and only in 3 of the 20 CSF of the UIAs samples).

According to the best knowledge, this is the first study evaluating the CSF sICAM-1 concentrations in CNS brain tumors or UIAs. So far sICAM-1 concentrations have been analyzed only in CSF of human African trypanosomiasis individuals [17], thus we could not estimate the concentrations of targeted protein and we performed the experiment with the dilution factor recommended by the manufacturer. Based on the obtained results, we hypothesize, that for sICAM-1 analysis in the CSF we did not use the appropriate dilution factor and the best way to test the sICAM-1 concentrations in the CSF is to analyze undiluted samples. However, we could not re-test undiluted CSF samples because of lack of material in order to confirm this Figure 4. Despite the inappropriate dilution factor, the second reason of failed sICAM-1 concentrations results in the CSF might be the sensitivity of the assay kit (there is a possibility that more sensitive assays are needed).

Current study revealed, that regardless of the parameter tested and the patients group, statistical differences were found between concentrations obtained in CSF compared to values obtained in serum for all proteins measured. Significant differences were found between the study groups in CSF for IL-8, while in serum differences were obtained for CCL2 and sICAM-1. Our findings indicate altogether, that for individual biomarkers (IL-8 and CCL2, sICAM-1) the appropriate material, respectively CSF or serum, should be chosen and quantitatively tested.

Our study revealed statistically increased CSF IL-8 concentrations in CNS brain tumors as compared to non tumoral UIAs, which indicate, that this chemokine may be synthesized within the CNS tissue during astrocytic tumors development and IL-8-related neuroinflammation can have a significant influence on glioblastoma (GBM) progression. Enhanced secretion of IL-8 by glioma cells was also reported by *Yeung et al.* [18]. IL-8 was also inversely correlated with GBM patients survival [19]. GBM cells, which secrete IL-8, promote angiogenesis and microvascular endothelial permeability [6]. IL-8 can also regulate GBM associated “cancer stem cells’ (CSCs) and was related with tumor grade in astrocytic neoplasms [20]. IL-8 induces growth and migration of CSCs via receptors CXCR1 and CXCR2. Observations concerning human microvascular ECs revealed that the blockade of either receptor decreases IL-8-related chemotaxis. *de la* *Iglesia et al.* showed, that in PTEN-deficient glioblastoma cells, repression of IL-8 can inhibit glioma cells proliferation and invasiveness [21].

In current study the analysis of clinical usefulness of CSF IL-8 revealed its clinical significance. Moreover, for CSF IL-8 we determined values of 80% sensitivity for detecting astrocytic brain tumors patients and 80% specificity for distinguishing them from non tumoral individuals, which renders IL-8 as an almost ideal biomarker.

CCL2 originally has been purified to homogeneity and then cDNA cloned from the glioma cell line [3,5]. The production of CCL2 by different types of human malignant glioma cell lines has been well documented. It was also noted, that malignant glioma cells produce more CCL2 as compared to other tumor cells lines, such as melanoma or malignant fibrous histiocytoma [22].

According to the best knowledge, our study is the second, which evaluated CCL2 concentrations in the CSF of patients with CNS brain tumors. However, results of our group are in disagreement with the reports of *Kuratsu et al., which* revealed that CCL2 concentrations in CSF samples from malignant glioma subjects were statistically higher in comparison to individuals with benign glioma as well as compared to non-tumoral patients[22]*.* We did not reveal significant differences for CSF CCL2 between brain tumors group as compared to patients with no tumor. Moreover, CCL2 concentrations revealed a tendency to be lower in brain tumors as compared to non-tumoral individuals.In our opinion the discrepancies between the results of these two studies may result from: 1) different antibodies applied by means of ELISA (*Kuratsu et al.* used rabbit polyclonal antibody and mouse monoclonal antibody (clone E11) against human CCL2; our group applied ready to use ELISA plate coated with a monoclonal antibody specific for human and recombinant CCL2 from well-known vendor (R&D Systems); and/or 2) not homogenous CSF samples (*Kuratsu et al.* collected CSF to analysis from different CNS spaces (lumbar interspace/cisternography by drainage during the surgery/directly from cisterns or brain ventricles), while our group collected all CSF samples from supratentorial subarachnoid space; and/or 3) different number of non tumoral subjects (*Kuratsu et al.* – N=7, our group – N=20). We have one more explanation why in our study CSF CCL2 concentrations were lower as compared to non-tumoral subjects: this may be due to fact, that CNS astrocytic tumors, which grow inside the tumor mass, may not have a possibility to contact with the CSF and thus CCL2 cannot be release into the cerebrospinal fluid.

In our study, serum CCL2 and sICAM-1 concentrations were significantly decreased in CNS tumors as compared to non-tumoral UIAs subjects. From both proteins tested in the serum higher clinical significance revealed CCL2. However, the CNS is actually still not recognized as an immune privileged site, there is no strong evidence in the available literature that the protective brain barriers are meaningfully altered in malignant gliomas. Therefore, blood CNS tumor biomarkers, defined as a measurable indicators of the diseases, its progress, and response to therapeutic intervention, are of great clinical value. Biomarkers, which may be detect and measure in the peripheral blood, are of important interests due to the fact, that blood is easy to obtain and blood collection procedure is not as traumatic as lumbar puncture and involves only a momentary discomfort to the patient.

*Advantages and disadvantages of the study*

The first advantage of our study was the evaluation of proteins tested both, in the CSF as well as in the peripheral blood. Thus, to exclude possible fluctuations of the BBB and the blood-CSF barrier, we could refer obtained results in CSF to values in serum. The second strong point of our research was the evaluation of proteins tested in CSF collected from the same CNS interspace, so these samples were relatively homogenous.

Our study has also few disadvantages: 1) small study group, which we try to compensate by equal number of participants included to tumoral patients (N=20) and non-tumoral subjects (N=20); 2) values obtained in patients with astrocytic tumors should be compared with another primary CNS tumors (e.g. meningeal tumors) as well as metastatic tumors (similar study in progress); 3) analysis of the clinical utility of proteins tested is limited, due to the efficiency of a test changes with the prevalence of the disease and the position of the threshold value (if the sensitivity related to a selected cut-off value is higher than the specificity, the efficiency will increase with increasing prevalence of the disease; if the specificity related to a selected threshold value is higher than the sensitivity, the efficiency will decrease with increasing prevalence of the disease). In our study the number of brain tumors subjects was equal to number of subjects from comparative group, so the test analysis did not reflect the prevalence of astrocytic brain tumors in the population.

**Conclusion**

In conclusion, our results support that CSF IL-8 concentrations could play a crucial role in predicting the presence of astrocytic tumors. However, there is need for further studies, if IL-8 may also be recognized as a helpful biomarker assessing patients response to the applied therapy. From biomarkers tested in the blood, CCL2 seems to have clinical utility, therefore for individual biomarkers (IL-8 and CCL2, sICAM-1) the appropriate material, respectively CSF or serum, should be chosen and quantitatively tested. To sum up – increased CSF IL-8 with decreased serum CCL2 give a biomarkers pattern, which with combination with conventional therapeutic strategy may be helpful in management of CNS astrocytic brain tumors.

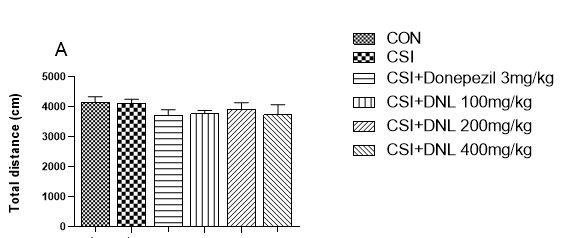
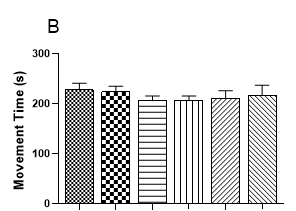
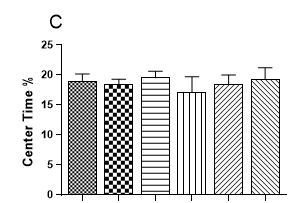
Author Contribution

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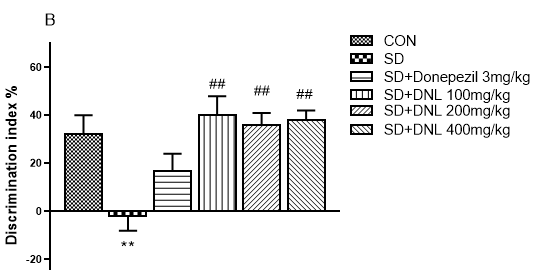
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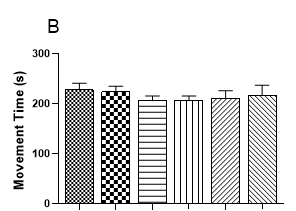
**Figure captions**

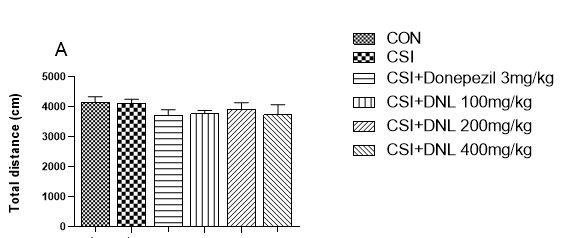
**Figure 1:** The effect of DNL on locomotor activities: total distance(A), moving time(B), center time(C) in the open field test after CSI for two weeks in mice. n=10-12. Values are mean ±SEM. \**p* <0.05 vs. the Con group, \*\**p* <0.01 vs. the Con group; #*p* <0.05 vs. the CSI group, ##*p* <0.01 vs. the CSI group.



**Figure 2:** The effect of DNL on the discrimination index (DI) during the testing session in the NOR task after CSI for two weeks in mice. N=10-12. Values are mean ±SEM . \*\**p* < 0.01 vs. the Con group; ##*p* < 0.01 vs. the CSI group.

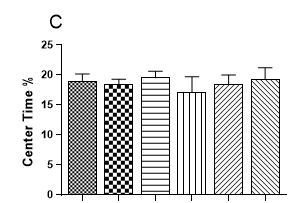


**Figure 3:** The effect of DNL on the discrimination index (DI) during the testing session in the NOR task after CSI for two weeks in mice. N=10-12. Values are mean ±SEM . \*\**p* < 0.01 vs. the Con group; ##*p* < 0.01 vs. the CSI group.

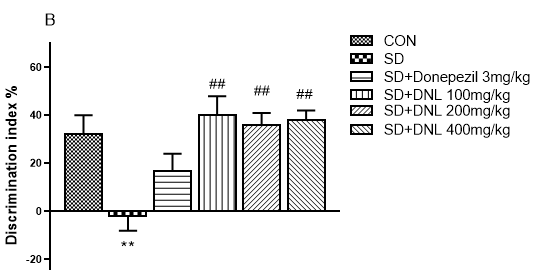


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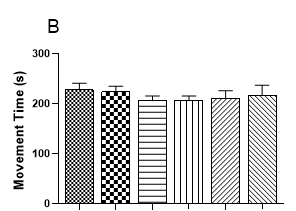
**Schemes**



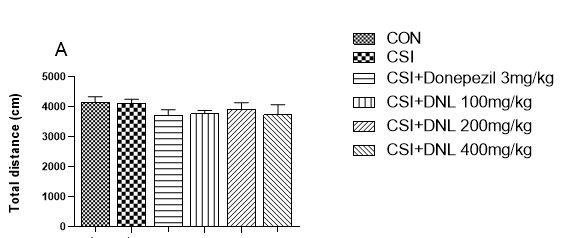
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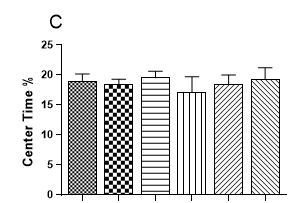


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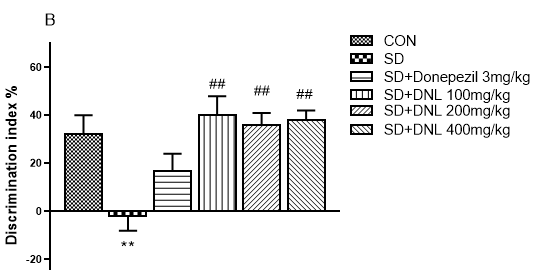


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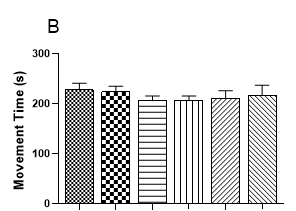
**Charts**



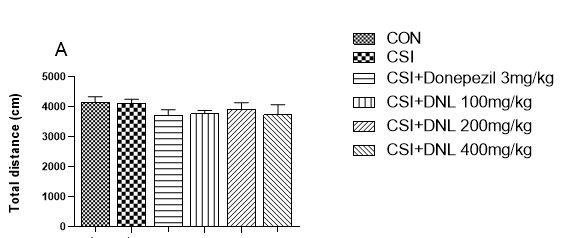
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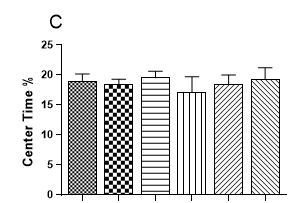


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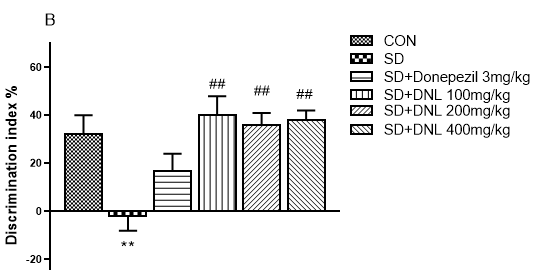


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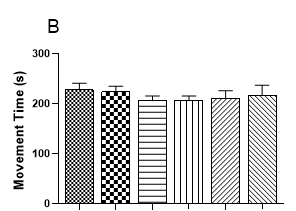
**Structures**



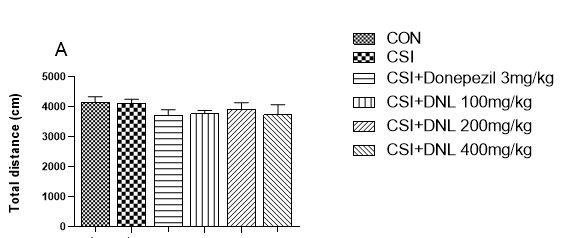
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