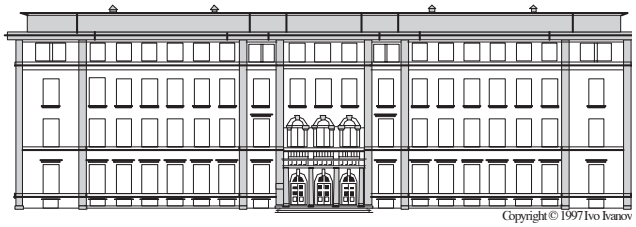

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Investigation Report on Project:
Cytoprotective effects of creatine (conventional or optimised [KreVitalin®]) in an in vitro model of NMDA induced-neurotoxicity

Experimental design

Excitotoxicity is a common mechanism implicated in the pathogenesis of diverse neurodegenerative disorders. The excessive release of excitatory amino-acid transmitters (e.g. glutamate) which is common in trauma, stroke, reperfusion injury etc. evokes neuronal cell death through activation of excitatory amino acid receptors, which in turn recruit signal cascades leading to increased intracellular calcium levels and cell death. Among these receptors the NMDA-subtype, a ligand-activated ionophore, plays crucial role in neuronal cell death and thus NMDA-induced cytotoxicity is a standard model for assessment of neurotoxicity and its modulation. The neuroprotective effects of both conventional and buffered creatine formulations was tested in a comparative fashion using a model of NMDA (N-methyl-D-aspartate)-induced cytotoxicity in human neuroblastoma-derived SH-SY5Y cells. A preliminary experiment showed that both conventional and optimised formulation were practically devoid of cytotoxic effects in this cell line, within a concentration range of 0.1-1 mmol/L.

Experimental protocol

Exponentially growing SH-SY5Y cells were plated in 96-well microplates and after a 24 h adaptation period they were exposed to NMDA (at 1000, 1500, 2000 or 2500 µmol/L), alone or in combination with 0.2 or 1 mmol/L creatine (conventional or optimised). Following a 72 h continuous exposure the cellular viability was assessed using the MTT-dye reduction assay.

Results

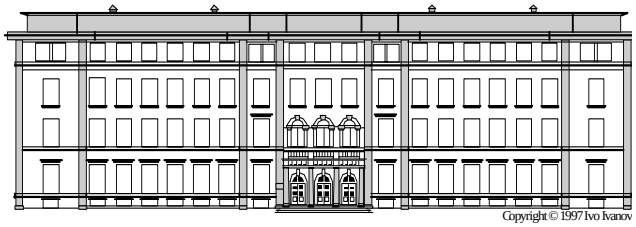
The neurotoxin NMDA evoked strong concentration-dependent reduction of cell viability, whereby at the lowest level evaluated (1000 µmol/L) there were approximately 80 % viable cells. At the highest concentration of 2500 µmol/L NMDA the fraction of viable cells was reduced by more than 50 %.

In line with the established neuroprotective properties of creatine in vivo and in vitro the co-administration of the conventional preparation was consistent with less pronounced neurotoxicity as compared to the sole application of NMDA. The neuroprotective effects were more pronounced at the higher creatine level investigated (1 mmol/L) (Table 1.; Figure 1.).

The combined treatment of SH-SY5Y cells with NMDA and the optimised creatine led to even more prominent, amelioration of the established excitotoxicity. In all treatment groups the combination of NMDA+optimised creatine was associated with significantly higher cell viability as compared to the effects of the neurotoxin alone (Table 2; Figure 2.).

Conclusions:

Although the mechanistic aspects of this neuroprotection need further clarification it is most probably an outcome of creatine's effects on cellular bio-energetics. Maintaining higher ATP and macroergic phosphate levels renders neuronal cells more robust and enhances their survival, previous investigations also suggest that creatine supplementation conditions lower responsiveness of neurons to pro-apoptotic stimuli. As with the nephroprotection study the superior cytoprotective effects of the processed creatine formulation vs the conventional one could be



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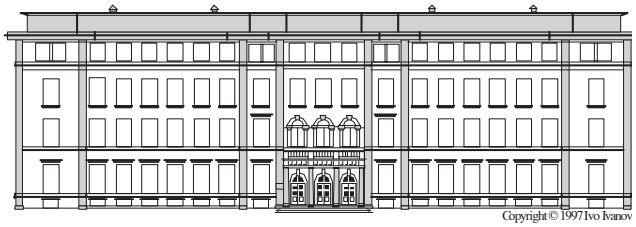
principally ascribed to the better stability of the former, which is a crucial prerequisite for optimal activity in vitro.

Project coordinator
and senior researcher: ……..
Asst. Prof. Georgi Tsvetanov Momekov, MPharm, PhD,

Lab Technician: ……..
Mrs. Theodora Atanassova, BSc

Sofia August 25th 2007



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Appendix Cytoprotective effects of creatine (conventional or optimised) in an in vitro model of NMDA induced-neurotoxicity . Experimental data.

Table 1. Cytoprotective effects of the optimised creatine formulation against NMDA-induced excitotoxicity in SH-SY5Y human cells, as assessed by the MTT- assay after 72 h incubation.

Treatment group	% of viable cells		Protection index
	Mean	sd	
Untreated control	**100.0**	**3.1**	**-**
NMDA 1000 μmol/L	**82.0***	**2.0**	**-**
+ 0.2 mmol/L creatine	**86.4*#**	**1.1**	**1.05**
+ 1 mmol/L creatine	**88.1*#**	**1.4**	**1.07**
NMDA 1500 μmol/L	**74.8***	**1.5**	**-**
+ 0.2 mmol/L creatine	**80.1*#**	**4.2**	**1.07**
+ 1 mmol/L creatine	**88.4*#**	**1.4**	**1.18**
NMDA 2000 μmol/L	**56.0***	**4.6**	**-**
+ 0.2 mmol/L creatine	**61.6***	**0.9**	**1.10**
+ 1 mmol/L creatine	**68.3*#**	**1.5**	**1.22**
NMDA 2500 μmol/L	**48.4***	**2.1**	**-**
+ 0.2 mmol/L creatine	**56.2*#**	**5.6**	**1.16**
+ 1 mmol/L creatine	**65.9*#**	**0.4**	**1.36**

*Statistically significant ($p<0.05$) vs. the untreated control; # Statistically significant ($p<0.05$) vs. NMDA administered alone (Student's t-test).

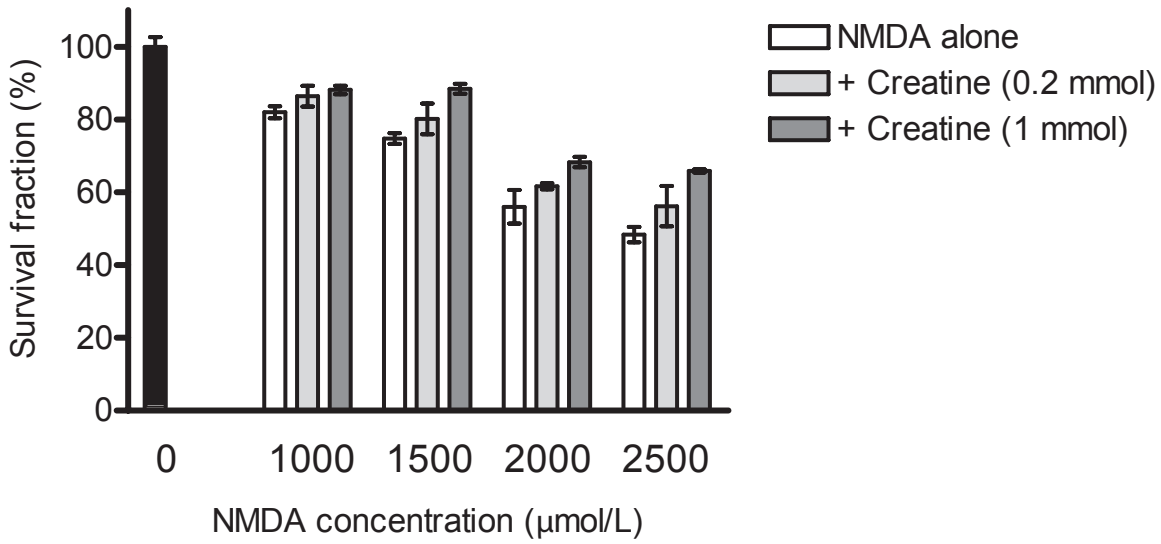

Fig. 1. Cytoprotective effects of the conventional creatine formulation against NMDA-induced excitotoxicity in SH-SY5Y cells, as assessed by the MTT-dye reduction assay after 72 h incubation. Each column represents the arithmetic mean ± sd (n=6).

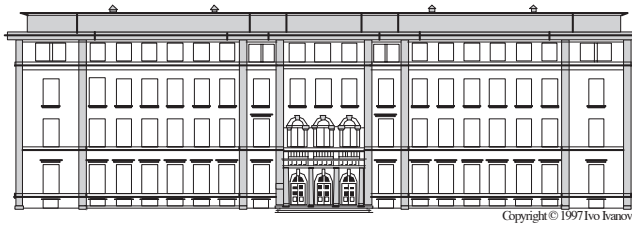

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Table 2. Cytoprotective effects of the optimised creatine formulation against NMDA-induced excitotoxicity in SH-SY5Y human cells, as assessed by the MTT-dye reduction assay after 72 h incubation.

Treatment group	% of viable cells		Protection index
	Mean	sd	
Untreated control	100.0	3.1	-
NMDA 1000 µmol/L	82.0*	2.0	-
+ 0.2 mmol/L Opti. creatine	92.4*#	5.7	1.13
+ 1 mmol/L Opti. creatine	101.3#	4.0	1.23
NMDA 1500 µmol/L	74.8*	1.5	-
+ 0.2 mmol/L Opti. creatine	85.0*#	4.2	1.14
+ 1 mmol/L Opti. creatine	93.0*#	2.7	1.24
NMDA 2000 µmol/L	56.0*	4.6	-
+ 0.2 mmol/L Opti. creatine	65.2*#	1.6	1.16
+ 1 mmol/L Opti. creatine	78.8*#	1.9	1.41
NMDA 2500 µmol/L	48.4*	2.1	-
+ 0.2 mmol/L Opti. creatine	63.8*#	1.8	1.32
+ 1 mmol/L Opti. creatine	69.0*#	2.6	1.43

* Statistically significant ($p<0.05$) vs. the untreated control; # Statistically significant ($p<0.05$) vs. NMDA administered alone (Student's t-test).

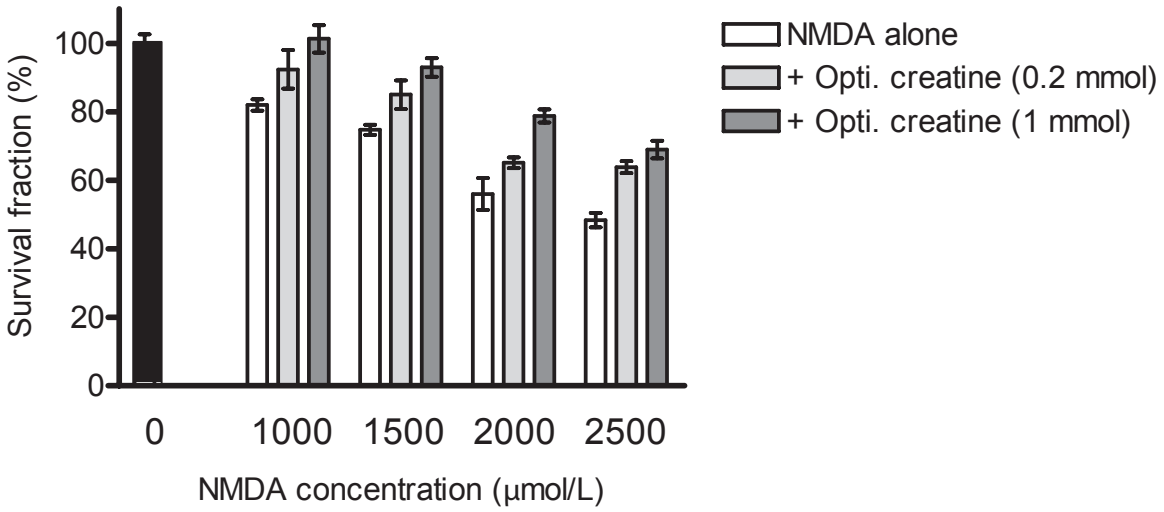

Fig. 2. Cytoprotective effects of the optimised creatine formulation against NMDA-induced excitotoxicity in SH-SY5Y cells, as assessed by the MTT-dye reduction assay after 72 h incubation. Each column represents the arithmetic mean ± sd (n=6).

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