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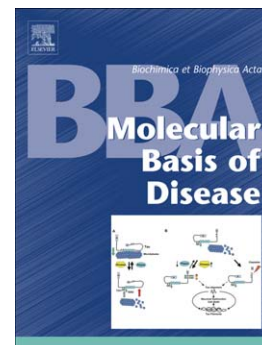
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**Gene therapy of Hunter syndrome: evaluation of the efficiency of muscle electro gene transfer for the production and release of recombinant iduronate-2-sulfatase (IDS)**

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## SUMMARY

Mucopolysaccharidosis type II (MPSII) is an inherited disorder due to a deficiency of the lysosomal enzyme iduronate-2-sulfatase (IDS). The disease is characterized by a considerable deposition of heparan- and dermatan-sulfate, causing a general impairment of physiological functions.

Most of the therapeutic protocols proposed so far are mainly based upon enzyme replacement therapy which is very expensive. There is a requirement for an alternative approach, and to this aim, we evaluated the feasibility of muscle electro gene transfer (EGT) performed in the IDS-knockout (IDS-ko) mouse model.

EGT is a highly efficient method of delivering exogenous molecules into different tissues. More recently, pre-treatment with bovine hyaluronidase has shown to further improve transfection efficiency of muscle EGT.

We here show that, by applying such procedure, IDS was very efficiently produced inside the muscle. However, no induced IDS activity was measured in the IDS-ko mice plasma, in contrast to matched healthy controls. In the same samples, an anticipated and rapidly increasing immune response against the recombinant protein was observed in the IDS-ko vs control mice, although reaching the same levels at 5 weeks post-injection. Additional experiments performed on healthy mice showed a significant contribution of hyaluronidase pre-treatment in increasing the immune response.

## INTRODUCTION

Mucopolysaccharidosis type II (MPS II), Hunter syndrome, [MIM 309900] is a rare, X-linked lysosomal storage disorder (LSD) due to the deficiency of the enzyme iduronate-2-sulfatase (IDS, EC 3.1.6.13) involved in the lysosomal catabolism of heparan- and dermatan-sulfate. Two major forms of the disease, severe and attenuated, are now recognized as having different progression and life expectancy. The pathology is mainly characterized by the accumulation of undegraded glycosaminoglycans (GAG) in spleen, liver, heart, kidney, bone and joints, and in the most severe forms it also involves the central nervous system leading to progressive neurodegeneration.

Only palliative procedures and symptomatic therapies are currently available to treat this disorder. Haematopoietic stem cell transplantation which has been successful for some other forms of MPS [1-3], is ineffective in Hunter disease [4]. After the recent conclusion of the phase III clinical trial [5], enzyme replacement therapy (ERT) has become the elective therapy, although it is expensive and, in addition, details of long term efficacy are not yet available.

Alternative therapeutic strategies have been evaluated including gene transfer, mainly using viral vectors [6-11], together with a somatic cell therapy approach [12].

Electro gene transfer (EGT) is a highly efficient method of delivering exogenous molecules into cells. It has been used to introduce DNA into various mouse and rat tissues [13-18] and it has also been successfully proposed to improve transfection of skeletal muscle with plasmid DNA [16, 19-25]. In fact, although muscle represents a very accessible tissue, and therefore a good gene transfer target, many drawbacks in obtaining high transfection efficiencies had been previously described by using direct injection [26, 27]. Attempts to further improve EGT efficiency have included pre-treatment of the muscle with hyaluronidase (HYA) [28-30].

In this paper we present our results on muscle electro gene transfer applied to the MPS II mouse model [31] and to control mice. The aim was to evaluate the feasibility and efficiency of this procedure when applied to the production and release of a systemic therapeutic protein, iduronate-2-sulfatase. The use of the Hunter disease-mouse model, characterized by null IDS activity in all the organ systems, allowed a clearer evaluation.

## MATERIALS AND METHODS

### *Plasmid construction*

All cloning procedures were performed following standard techniques [32]. Human IDS cDNA (kindly provided by J Hopwood, Children, Youth and Women's Health Service, Adelaide,

Australia) had been previously modified by Bielicki et al in 1993 [33] by adding the 45 bp of the rat pre-proinsulin leader sequence. The cDNA had been cloned by us in pVIJnsB [34] as previously described [22]. The resulting plasmid, called pVIJIDS, carries the IDS cDNA sequence under CMV transcriptional control and bovine growth hormone polyA.

### *Mice*

C57BL6 control mice were purchased from Charles River Italia while IDS-knockout (IDS-ko) mice were obtained from heterozygous founders kindly provided by J. Muenzer (Chapel Hill, USA).

The IDS-ko mouse model was created by replacing exon 4 and part of exon 5 of the IDS gene with the neomycin resistance gene [35]. As previously reported [12], knockout mice show no detectable plasma and tissue IDS activity.

In the first experiment a total of 9 IDS-ko and 12 healthy mice were treated with EGT after pre-treatment with bovine hyaluronidase. All animals were injected with 50 µg of plasmid DNA and seven further healthy mice were injected with PBS only, as controls. Animals treated at 12-16 weeks of age were euthanized at 3 time-points 1, 2, and 5 weeks post-injection and blood and tissue samples were collected and analyzed.

In a second study, a total of 44 healthy mice were treated with the same procedure. Only half of the mice were pre-treated with hyaluronidase and animals were progressively euthanized 1, 2, 5 and 8 weeks later. Moreover, 18 mice not pre-treated with hyaluronidase were injected with 1 µg of plasmid DNA and monitored for 5 weeks post-treatment. Blood and tissue samples were collected and analyzed.

All animal experimentation was performed in accordance to National and International Animal Ethic Guidelines.

### *Electro Gene Transfer (EGT)*

EGT was performed on anaesthetized mice by injecting 50 µg of plasmid DNA per animal diluted in 50 µl total saline into the quadriceps muscles. The mouse muscle was surgically exposed, injected and then immediately electro-stimulated by inserting needle steel electrodes around the injection site. The electric field was applied in a pulsed form by using a Pulsar 6bp-a/s bipolar stimulator and pulses were monitored with a digital oscilloscope. Mice were electrically stimulated with ten trains of 1000 square bipolar pulses, maintaining current control at 75 mA, delivered every other second, for a total duration of 20,1 seconds. The frequency was 1000 Hz. Type VI-S hyaluronidase from bovine testis (36 units per animal), resuspended in 50 µl sterile saline, was injected into the muscle 45 minutes before EGT, as described by Mennuni and co-workers (2002).

*IDS enzyme assay*

IDS activity was evaluated by a fluorimetric assay [36] requiring the use of the substrate 4-methylumbelliferyl- $\alpha$ -iduronide-2-sulphate (Moscerdam Substrates, Erasmus University, Rotterdam, the Netherlands). The assay was performed according to the manufacturer's instructions. Briefly, 10  $\mu$ g of protein per sample or 2  $\mu$ l of plasma were assayed by adding 20  $\mu$ l of 1.25 mM substrate and incubating at 37 °C for 4 hours. Afterwards, 40  $\mu$ l of double concentrated McIlvains phosphate/citrate buffer and 10  $\mu$ l of LEBT (Lysosomal Enzymes purified from Bovine Testis) were added to each sample and incubated at 37 °C for further 24 hours. The reaction was stopped by adding 1420  $\mu$ l per sample of stop buffer (0.5 M NaHCO<sub>3</sub>/0.5 M Na<sub>2</sub>CO<sub>3</sub> pH 10.7, 0.025% Triton X-100); 4-methylumbelliferone (Sigma-Aldrich, Milano, Italy) was used as standard. Fluorescence was measured at 355/460 nm excitation/emission in a Victor 2 1420 Multilabel Counter Fluorometer (Wallac-Perkin Elmer). Enzyme activity was given as nmoles of substrate hydrolysed in 4 hours per mg of total protein or per ml of plasma [nmol/4h/mg or nmol/4h/ml]. Tissue samples were first homogenized in lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA pH 8.0, 0.1% Triton X-100) and, following centrifugation, the supernatants were recovered. All samples were evaluated for protein content by using the BIORAD protocol. Plasma samples were obtained from heparinized blood, microcentrifuged at 3000 rpm for 20 minutes.

*Anti-iduronate-2-sulfatase antibodies determination*

Anti-iduronate-2-sulfatase antibodies were measured by using an immunoprecipitation assay [12]: 5  $\mu$ l of mouse plasma was diluted in 40 mM Tris-HCl, pH 8.0 to a final volume of 60  $\mu$ l, mixed with 80 nmol/4h of iduronate-2-sulfatase collected from over-expressing C2C12 cell medium and incubated at 4° C overnight. The day after, samples were incubated with 20  $\mu$ l of Pansorbin protein A (200 mg/ml) (Calbiochem, INALCO, Milano, Italy) and centrifuged to pellet iduronate-2-sulfatase bound to Pansorbin in the presence of anti-iduronate-2-sulfatase antibodies. The unbound enzyme in the supernatant was measured with the fluorometric assay. Antibody titre was expressed as percentage of IDS immunoprecipitated by the antibodies, with respect to the initial IDS content. Negative control was obtained by incubation of an equal amount of plasma from ko untreated mice. For the positive control 80 nmol/4h of IDS were incubated with 9  $\mu$ g total of a goat anti-human IDS polyclonal antibody (kindly provided by Shire-TKT, Cambridge, USA) in order to completely abolish the enzyme activity.

*Tissue glycosaminoglycan content*

Tissue GAG content (expressed as  $\mu\text{g}$  of GAG/mg of protein) was measured as previously suggested [37]. Before performing the assay, tissues were lyophilised, resuspended and homogenized in 0.9% NaCl + 0.2% Triton X-100. Samples were then shaken overnight at  $4^{\circ}\text{C}$  and microcentrifuged for 5' at 3000 rpm. Supernatants were collected and kept at  $-80^{\circ}\text{C}$  until analysis. 50  $\mu\text{l}$  of blank (water), calibrators (chondroitin sulfate C, Sigma-Aldrich, Milano, Italy) and samples in duplicate were incubated with 50  $\mu\text{l}$  of 8 M Guanidine-hydrochloride for 15 minutes at room temperature, with 50  $\mu\text{l}$  of SAT solution (0.3% sulfuric acid, 0.75% Triton X-100) for 15 minutes and with a solution of Alcyan blue 0.038% overnight. Afterwards, samples were centrifuged and rinsed with a DMSO washing solution (40% DMSO, 0,05% Magnesium chloride). After centrifugation, pellets were resuspended in Gu-Prop (4M Guanidine-hydrochloride, 33% Isopropanol, 0.25% Tiron X-100) and absorbance was measured at 620 nm.

*Statistical analysis*

Data represent the average of different samples, the number of which is indicated under each figure. Bars represent SEM (mean standard error). Statistical comparisons of experimental groups were performed using a Student's t-test. *p* values less than 0,05 were considered statistically significant.

## RESULTS

*Experimental plan.* We first evaluated the EGT procedure for the release of iduronate-2-sulfatase in hyaluronidase pre-treated IDS-ko and control mice. In a second experiment, performed in healthy control mice, we compared the efficacy of the procedure in animals pre-treated and not pre-treated with hyaluronidase.

### EGT in hyaluronidase pre-treated IDS-ko mice

#### *IDS activity induced in quadriceps muscles*

Our first evaluation was performed in a total of 9 IDS-ko and 12 healthy mice treated with EGT (after injection of 50 µg of the plasmid DNA pvIJIDS per animal) and pre-treated with bovine HYA. Nine further healthy animals underwent EGT following injection of PBS only, and were used as controls. Mice were euthanized at 3 time-points: 1, 2 and 5 weeks post-injection. Quadriceps were removed, homogenized and evaluated for IDS activity by performing a fluorometric assay, and the results are shown in Figure 1. The basal enzyme level reported ( $9.8 \pm 1.9$  nmol/4h/mg) was calculated in quadriceps muscles from PBS injected healthy animals (indicated as UT wt), while no basal IDS activity was measured in the IDS-ko mice (data not shown). The difference between electro-injected muscles and the basal level was assumed to represent enzyme activity due to the recombinant protein. In all groups, a significantly increased activity ( $p < 0.0001$ ) of at least six fold ( $63.8 \pm 10.8$  nmol/4h/mg) with respect to the basal value ( $9.8 \pm 1.9$  nmol/4h/mg) was detected, reaching a level 24-fold higher ( $234.9 \pm 46.5$  nmol/4h/mg) than baseline in healthy mice euthanized 5 weeks post-treatment.

#### *IDS activity measured in plasma*

In the same animals we evaluated plasma IDS activity (Figure 2). Since our previous experience showed that IDS plasma level in adult mice maintains constant with time (laboratory data, not shown), we took as comparing level for the present experiment the mean activity detected before treatment (basal level, time 0,  $74.8 \pm 14.8$  nmol/4h/ml). A higher IDS activity with respect to the control value was detected in the healthy mice 2 and 5 weeks post-treatment, with a maximum increment of about 30 units (+34%) ( $100.4 \pm 32.1$  nmol/4h/ml,  $p < 0.05$ ) at 5 weeks; in contrast no induced activity was measured in the IDS-ko animals (not shown in the figure).

#### *Anti-IDS immune response*

The amount of specific anti-IDS antibodies raised by the treatment was evaluated in the animal's

plasma. The analysis was performed in both ko and wt mice at different time-points, by using an immunoprecipitation assay. Starting from a known IDS activity, we evaluated the residual activity following immunoprecipitation with the plasma of the treated animals. Results, given as percentage (%) of IDS activity neutralized by the plasma antibodies, are reported in Figure 3 and show an increasing amount of neutralizing antibodies from 1 to 5 weeks post-treatment. In particular, a considerably higher immune response was detected in IDS-ko mice with respect to controls at 2 weeks (53% vs 11% of IDS immunoprecipitated,  $p < 0.001$ ).

#### *Tissue IDS activity and GAG content*

IDS activity was also measured in liver, spleen, kidney, lung, heart and controlateral quadricep muscle of the treated IDS-ko mice. Enzyme activity detected was around 0 nmol/4h/mg of protein (data not shown). In the same organs glycosaminoglycan content measured 5 weeks post-treatment did not decrease with respect to untreated tissues (data not shown).

### **EGT in hyaluronidase pre-treated and not pre-treated control mice**

#### *IDS activity induced in quadricep muscles*

In a second experiment forty-four wild type animals were treated with the same procedure, half of which were pre-treated with HYA while half of them were not. Animals were progressively euthanized 1, 2, 5 and 8 weeks later. Figure 4 shows the enzyme activity detected in the mice quadriceps obtained post-euthanasia. As in the previous experiment, the difference between electro-injected muscles and the basal level was assumed to represent enzyme activity due to the recombinant protein. In most animals enzyme activity appeared significantly higher than baseline ( $9.8 \pm 1.9$  nmol/4h/mg, indicated as UT in the figure). Moreover, it was significantly higher in HYA pre-treated versus not pre-treated animals ( $p < 0.0001$ ), reaching a 20 fold increase ( $187.5 \pm 32.4$  nmol/4h/mg), with respect to basal level at 8 weeks.

#### *IDS activity measured in plasma*

Figure 5 reports plasma IDS levels measured in the EGT-treated animals. Mice were monitored weekly by retro-orbital bleeding. T0 represents pre-treatment values. A sudden increase of the activity was measured 1 week post-injection in both groups of samples (around +40%,  $p < 0.0001$ ). In the following time-points, up to 5 weeks, variable levels of activity were detected, some of which significantly increased compared to baseline, as indicated in the figure. The activities measured were quite similar between the two groups of animals. At 6, 7 and 8 weeks post-treatment samples collected from mice which were not treated with HYA showed a higher level of enzyme activity

with respect to the group of mice pre-treated (+39%, +24.5%, +29% respectively). In particular, such increase was statistically significant 6 and 7 weeks post-treatment ( $p < 0.01$ ).

#### *Anti-IDS immune response*

The appearance of specific anti-IDS antibodies was monitored weekly in the plasma of both animal groups (Figure 6). Neutralizing antibodies were measurable starting 2 weeks post-treatment and progressively increasing for the entire follow-up. The amount of antibodies detected in the samples derived from mice who underwent HYA pre-treatment was higher than that detected in the plasma obtained from animals who were not pre-treated with HYA, in the first group up to 50% of the IDS activity was immunoprecipitated by the antibodies. There was up to a four fold difference between the two groups at weeks 5 and 8 post-injection. Statistical analysis showed significant differences between the 3<sup>rd</sup> and 7<sup>th</sup> week of treatment ( $p < 0.005$  at weeks 3 and 4,  $p < 0.0001$  at weeks 5, 6, 7). Finally, a further decrease of the immune response was observed in a subsequent experiment, in which we injected 18 animals with 1  $\mu\text{g}$  of DNA. Results showed, 5 weeks post-treatment, a reduction from 10 to 5% of the IDS immunoprecipitated, compared to injection of 50  $\mu\text{g}$  of plasmid DNA without HYA pre-treatment. However, in this experiment we did not register a significant increase of plasma IDS activity with respect to basal level.

#### *IDS activity measured in tissues*

IDS activity was also measured in liver, spleen and controlateral quadriceps muscles of the treated healthy mice. Data shown in Table 1 present the comparison between the two groups of animals euthanized at 5 and 8 weeks post-injection. An increased enzyme activity was seen in the spleen of mice not pre-treated with hyaluronidase at 5 and 8 weeks post-treatment, in the spleen of HYA pre-treated animals at 5 weeks and in the liver of not-pre-treated mice at the same time. None of the increased values were statistically significant with respect to basal level.

## DISCUSSION

EGT is a common procedure for efficient targeted transduction of genes of interest. It has been applied to numerous tissues and organs in several animal models [13-18] and, in particular, it has been proposed to improve transfection of skeletal muscle with plasmid DNA [16, 19-25]. EGT has significantly improved muscle gene transduction, allowing a several fold increase in the amount of recombinant protein produced. Moreover, the procedure has been more recently optimized by pre-treatment of the muscle with hyaluronidase [28, 29], which has further increased its performance. We here evaluated the feasibility of utilizing muscle EGT for the efficient production and release of iduronate-2-sulfatase in the IDS-ko mouse model. IDS is a lysosomal enzyme involved in the catabolism of the glycosaminoglycans heparan and dermatan-sulfate. A deficiency in this enzyme activity is responsible for a genetic disease called Hunter syndrome or mucopolysaccharidosis type II (MPS II). The intravenous infusion of the recombinant enzyme has shown to improve the pathological phenotype; however, it remains extremely expensive (around 300.000 Euros/patient/year) and the search for new therapies is thus of primary importance.

It has recently been suggested that very small amounts of a lysosomal protein (3-5% of normal) would be sufficient for the effective treatment of a specific LSD [38, 39]. These data strongly encourages a gene therapy approach, albeit the amount of recombinant protein produced, released and taken up by the different tissues is very difficult to control.

Muscle EGT has never been assayed so far in animal models as a potential tool for gene therapy of lysosomal disorders. Previous works proposing muscle transduction for the treatment of these pathologies, had been performed using viral vectors, either adenovirus or adeno-associated virus, obtaining contradictory results. Enzyme release, uptake by the peripheral organs and a therapeutic effect were observed for Pompe [40] and Fabry diseases [41], while no release from the muscle was described for MPS VII [42] and MPS VI [43].

We here reported the experiments performed in the IDS-ko and wild type animal muscles, both pre-treated with hyaluronidase, to evaluate the efficacy of the procedure for IDS muscle production and its release into the plasma. The results showed an elevated production of the protein inside the muscle, but a limited release into the bloodstream and a strong anti-IDS immune response, which likely cleared the plasmatic fraction of the recombinant protein. To elucidate this sudden and strong immune reaction we performed an additional experiment on healthy control mice, half of which were pre-treated with hyaluronidase and half were not.

In the first experiment healthy and IDS-ko mice showed a very high induced enzyme activity in the muscle, up to 24 fold and 14 fold higher respectively than baseline wild type level. Since the

pathology presents a systemic involvement, to obtain therapeutic efficacy the recombinant protein needs to escape the muscle, enter the blood stream and reach the organs. Our experiments suggest that a small part of the large amount of IDS produced was released by the quadriceps since an increased IDS activity with respect to baseline level was detected in the plasma of the control animals. However, such activity showed values hardly detectable in the knock out mice plasma, where conversely an anticipated and stronger immune response with respect to the healthy animals was detected. This immune reaction started 2 weeks post-treatment and reached levels 5 fold higher than that measured in the control mice. In the latter an important immune response was detected at 5 weeks post-treatment. As a consequence, no induced activity was found in the organs analyzed from ko mice: liver, spleen, kidney, lung, heart and contralateral muscle.

Could this immune reaction be due to the procedure in itself? It is, in fact, well known that muscle *per se* is an important route of immunization: might hyaluronidase represent an adjuvant in such an immune reaction?

To address these questions we performed a second set of injections in healthy control mice, either pre-treated or not pre-treated with hyaluronidase. For this analysis we treated healthy animals because the reduced and slower anti-IDS antibodies production expected in these animals versus IDS-ko mice would have allowed a clearer evaluation of the immunogenicity of the procedure *per se*.

Comparison of the results obtained in the control animals pre-treated with hyaluronidase in the two experiments performed, showed a very good reproducibility of the procedure although carried on at two different times in two different batches of mice.

As previous papers had already shown [28, 29], hyaluronidase pre-treatment of the muscle significantly improves the outcome of EGT, with notable increases in the amount of protein produced inside the muscle. In our experiment, we observed an IDS level enhanced up to six fold in HYA pre-treated muscle versus not pre-treated. However, the present data suggest that the amount of recombinant protein released in the blood stream represents only a small amount of the IDS produced by the treated muscle, and we did not detect any significant differences between hyaluronidase pre-treated and not pre-treated animals up to 5 weeks post-injection. Starting at 6 weeks the IDS activity measured in the plasma derived from HYA-treated animals went back to baseline level, whilst the activity measured in not pre-treated mice remained at significantly higher levels with respect to baseline, until the end of the follow-up. This was shown to be related to the immune response against IDS: in pre-treated animals we detected a quicker and stronger immune reaction, which, starting at 5 weeks post-treatment, consistently maintained about four fold higher than that measured in the animals not pre-treated.

Previous experience by using recombinant IDS in preclinical experiments [12, 44] did not support for the protein an important immunogenic role in the short period. Therefore, present results suggest that most of the immune response here observed might have been caused by the administration route in itself, and that hyaluronidase might act as an adjuvant. In any case, analysis of the organs (liver, spleen, contralateral muscle) obtained from the euthanized animals at different time-points post-treatment, did not detect significant increases of IDS activity with respect to baseline levels, nor showed any differences between the two groups of animals.

Our data suggest that the muscle is a closed system in which only small amounts of the recombinant IDS produced can escape and reach the blood stream, and this seems to be independent from the amount of protein produced. This would seem to be different from what happens to proteins specifically directed or targeted to the blood stream, such as erythropoietin, interferon, interleukins and VEGF [25, 45-47] for which a good release had been described.

As for lysosomal enzymes in general, the results published so far seem to be quite disappointing and contradictory. The transgene was always delivered to the muscle by a viral vector, and only few authors demonstrate a release of the enzyme into the blood stream [39, 41]. Other authors rather suggest that the enzyme activity observed outside of the target muscle might be due to a spill over of the virus at the time of injection [42, 43].

In conclusion, we here confirmed that muscle electro gene transfer can very efficiently produce recombinant proteins inside the target muscle, in this case the iduronate-2-sulfatase; hyaluronidase pre-treatment significantly improved the outcome of the procedure.

However, we could not detect a high release of the protein from the producing muscle, either HYA pre-treated or not. Instead we observed an important anti-IDS immune reaction, which was increased by HYA pre-treatment.

Although EGT might be proposed as a tool to efficiently produce proteins used by the muscle, such as dystrophin for the treatment of muscular dystrophy, or for the efficient release of proteins specifically targeted to the circulation, such as erythropoietin or factor VIII or IX for the treatment of blood disorders, our study shows that EGT performed in the described conditions cannot successfully determine an efficient release of iduronate-2-sulfatase from the injected muscle, necessary for the treatment of Hunter disease.

The application of this procedure to pathologies caused by housekeeping genes, which involve most of the organ systems, whose treatment thus requires a body-wide distribution of the protein is an area in need of improvement, which might take advantage by technical modifications such as the use of external plate electrodes and/or the high voltage-low voltage combination, providing a more homogenous distribution of the electric field and a reduction of the tissue damage.

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ACCEPTED MANUSCRIPT

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**Table 1** IDS activity detected in mice tissues: comparison between hyaluronidase pre-treated and not pre-treated animals.

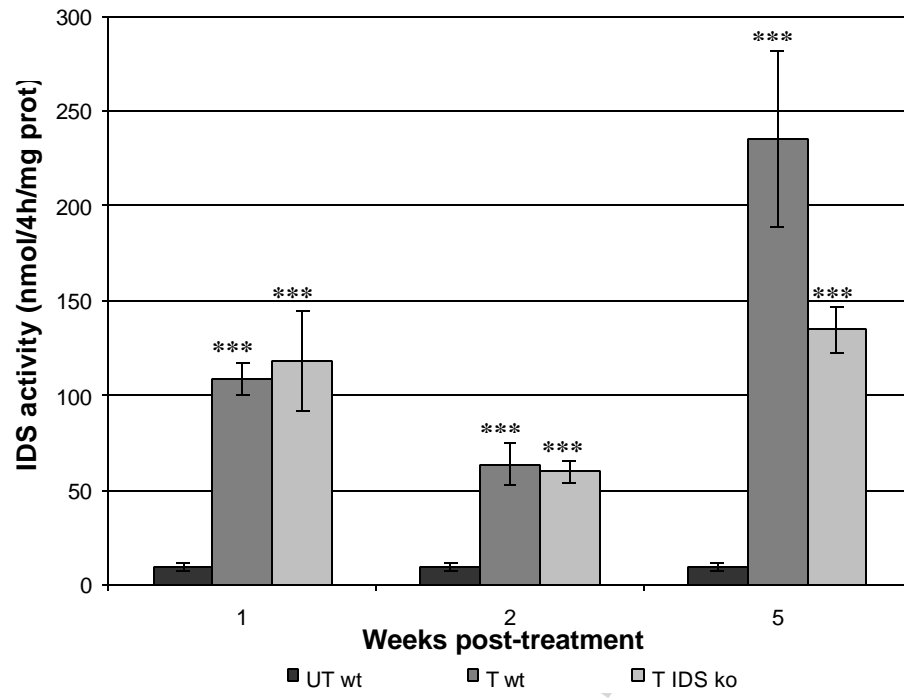
	<b>Basal level</b> a		5 w <sup>b</sup>	8 w <sup>b</sup>
<b>Liver</b>	<b>16,5±0,6</b>	- HYA + HYA	22,7±4,9 17,9±1,3	17,6±2,2 16,9±0,8
<b>Spleen</b>	<b>21,1±1,7</b>	- HYA + HYA	25,3±1,8 26,8±3,3	25,5±1,9 21,8±1,5
<b>Cl Muscle</b>	<b>9,8±1,9</b>	- HYA + HYA	10,8±3,7 8,4±1,3	7,3±2,7 8,8±1,2

<sup>a</sup> Tissue IDS activity in not treated healthy mice.

<sup>b</sup> Tissue IDS activity in treated healthy mice.

5 w and 8 w = weeks post-treatment, HYA = hyaluronidase, Cl: contralateral muscle.

IDS activity is given as nmol/4h/mg of protein

**Fig 1**

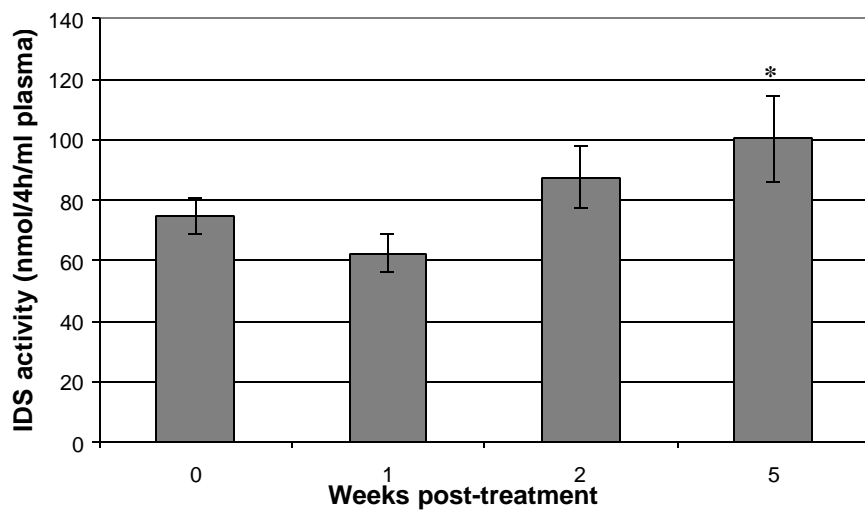


Fig 2

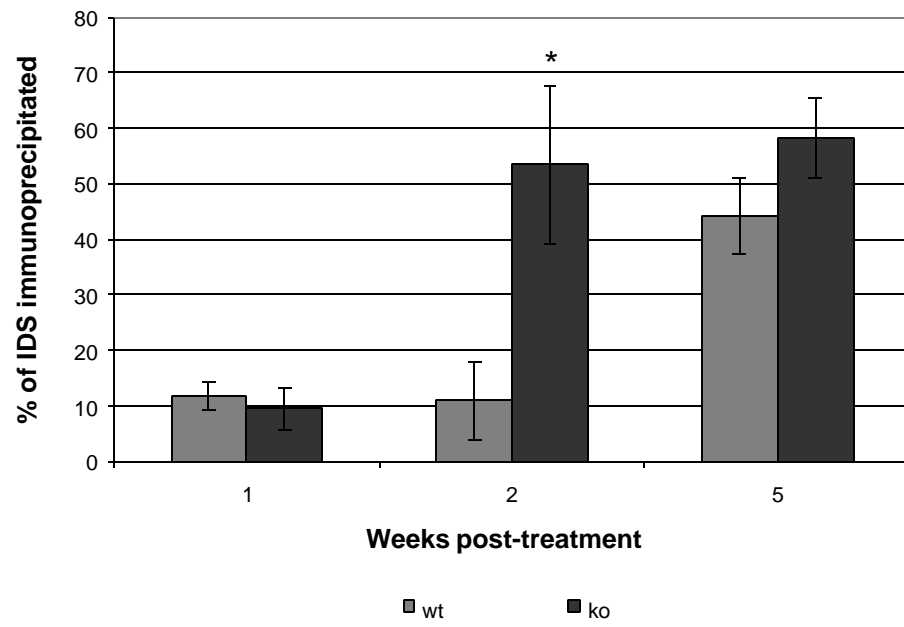


Fig 3

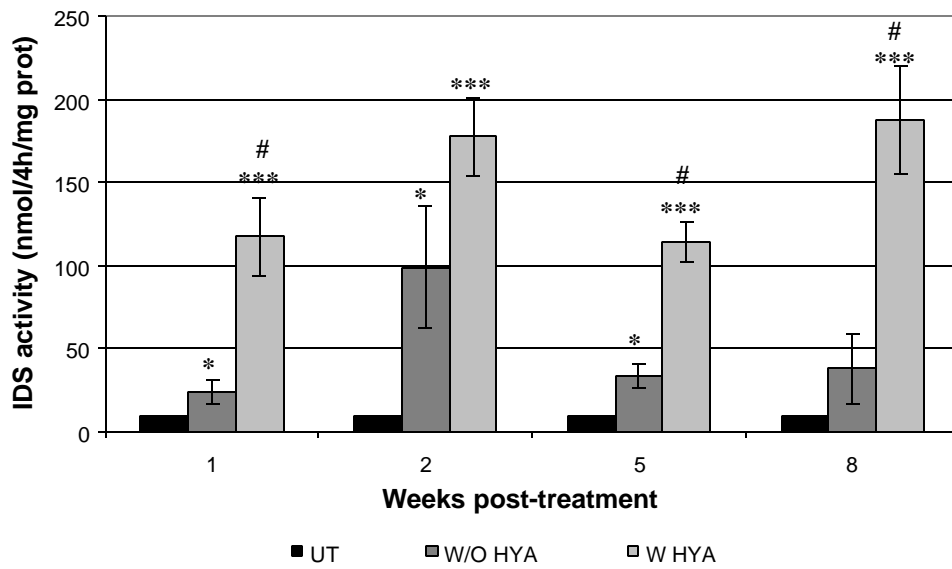


Fig 4

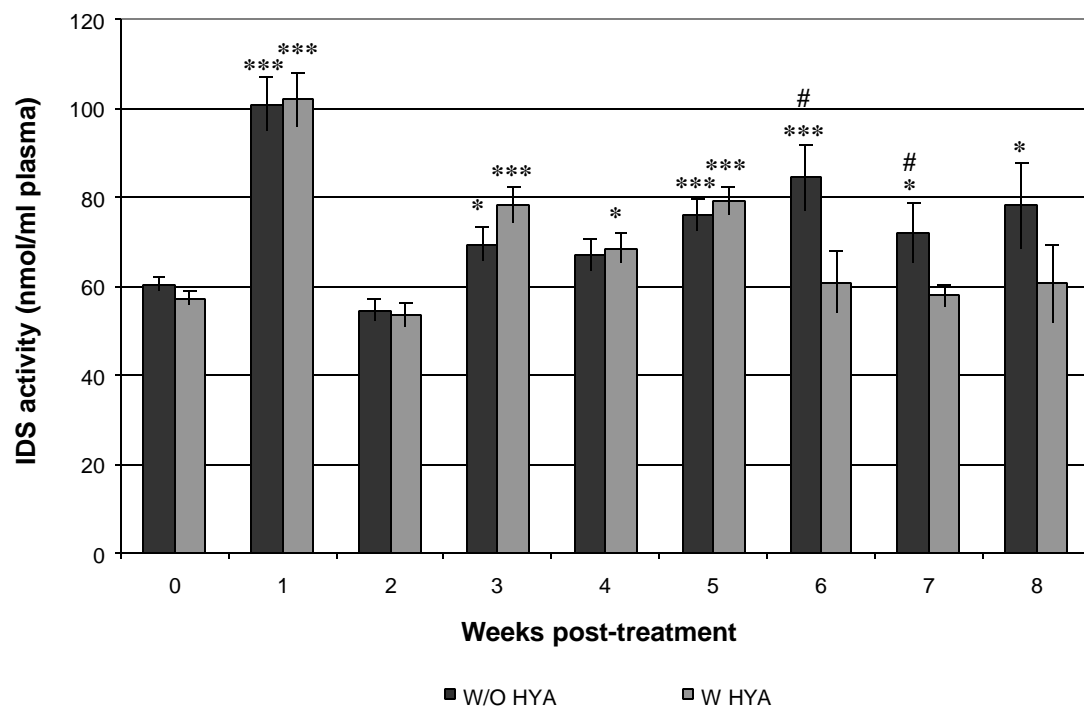


Fig 5

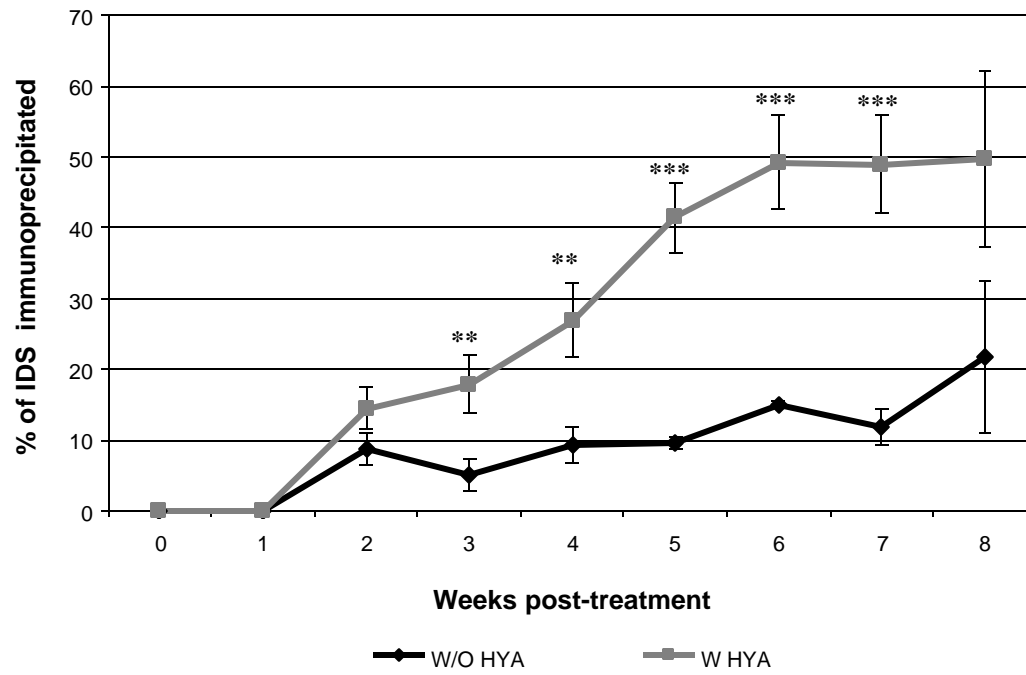


Fig 6