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# Single-dose extended-toxicity preclinical study on novel radiotracer formulations for use in the diagnosis of neuroendocrine tumor and neurodegenerative disorders

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## ABSTRACT

The use of [$^{18}$F]F-DOPA as an imaging agent for positron emission tomography (PET) studies of neurodegeneration and tumor detection is hindered by the chemical instability in its injectate formulation, which is also accompanied by injection-site reactions. Here we investigated novel F-DOPA lactate-based formulations that minimize formulation toxicity without compromising chemical stability. *In vivo* tolerability study on ND2 (lactate/$Na_2$EDTA/F-DOPA) and ND3 (acetic acid/F-DOPA) formulations in rats (i.v./i.m. 0.025-5 mg/kg) and mice (i.v. 50 mg/kg) was performed. Single-dose extended-toxicity study was performed in rats and mice in order to evaluate the acute and long-lasting response of the animals to the novel formulations. Toxicity related to ionizing radiation was not investigated because the stable isotope [$^{19}$F]F-DOPA was used instead of [$^{18}$F]F-DOPA which is the fluorine radioisotope that decays by b+ emission. The i.m. injection of ND3 (5 mg/kg) and ND3-vehicle to rats caused a local reaction characterized by up-regulation of the autophagic *Lc3* and *Bnip3*, apoptotic *Caspase 3*, *8* and *9*, mitochondrial *Pgc1α*, and inflammatory *Mapk3*, *Cgrp*, *TNFα* genes. *Mapk3* and *Pgc1α* were also up-regulated in the ND2 (5 mg/kg)-treated muscles. The i.v. injection of ND3 (5 mg/kg) caused a reduction of body weight in rats, after 14 days of follow-up; the ND3 (50 mg/kg) and the ND3-vehicle caused a loss of body weight of -17.8 ± 1% and -12.3 ± 2% *vs* controls, respectively, in mice. No effects were observed following the administration of ND2 and the ND2-vehicle in rats and mice. The F-DOPA lactate/$Na_2$EDTA-based formulation is better tolerated than the acetic acid-based formulation.

**KEYWORDS:** single-dose extended-toxicity, [$^{18}$F]F-DOPA, positron emission tomography-computerized tomography, neurodegeneration, neuroendocrine tumors, rodents.

## INTRODUCTION

The [$^{18}$F]F-DOPA formulations are largely used in the diagnosis of neurodegenerative disorders and neuroendocrine tumor (NET) by positron emission tomography (PET) [1-7].

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The prognosis for patients with NET is related to the tumor location and differentiation [8]. Improved diagnostic accuracy can be achieved in NET using somatostatin receptor scintigraphy-based methods such as Single-photon Emission Computed Tomography (SPECT), where the $^{111}$In-pentetreotide or other somatostatin analogues labeled with $^{99m}$Tc can be used because in some cases somatostatin receptors are up-regulated [9, 10]. The introduction of [$^{18}$F]F-DOPA using PET imaging and computed tomography (CT) allows to visualize a variety of neuroendocrine tumors [11, 12]. NET imaging agents also include $^{123}$I-metaiodobenzylguanidine and (4-Fluoro-3-iodobenzyl)guanidine [13, 14], $^{111}$In-pentetreotide, and PET radiotracers [18F]-fluorodeoxyglucose and somatostatin analogues ($^{68}$Ga-DOTATATE Netspot® Advanced Accelerator Applications USA, Inc.), indicated for localization of somatostatin receptor-positive neuroendocrine tumors in adult and pediatric patients [15].

Nevertheless, there is no universal SPECT or PET radiotracer for NET imaging. F-DOPA, as the other PET radiotracers, appears to be superior in diagnostic performance in a limited number of specific NET types, which are currently medullar thyroid cancer, catecholamine-producing tumors with a low aggressiveness and well-differentiated carcinoid tumors of the midgut, as well as in case of congenital hyper-insulinism [16]. Novel methods to improve F-DOPA synthesis from nucleophilic fluoride, to increase utilization as a PET imaging agent, have been reported [17-20].

The two available formulations of [$^{18}$F]F-DOPA (IASOdopa® (IASON GmbH) [21] and Dopacis® (CIS Bio International, IBA Molecular) [22] have some limitations related to the instability of the formulations and adverse site reactions [23-25], such as transient burning sensation and pain, following i.v. administration in humans [22, 26, 27]. However, the biological mechanisms responsible for these effects are not known.

We evaluated the *in vivo* effects of novel lactate buffer-based F-DOPA formulations, namely ND2, and one acetic acid-based F-DOPA formulation ND3 resembling the commercially available IASOdopa® for injectable use. The effects of these formulations were studied in local tolerability experiments following a single i.m., h.i., i.p. and s.c. injection within 24 h from the administration. Investigation with other administration routes, different from the main route used in human for the F-DOPA, is a regulatory requirement for radiotracer development [28]. Multiple administration route investigations are justified by the fact that the formulations under study are composed, other than by F-DOPA, also by acetic acid and lactate vehicles, which affect several organs and tissues including skeletal muscle, liver and renal tissues other than brain for instance. The effects of the F-DOPA formulations were therefore investigated in single i.v. dose extended-toxicity study. The aim of this protocol is the evaluation of acute toxicity of a single i.v. dose of drugs and the reversibility of the observed effects after 14 days of follow-up in rats and mice [28]. The different formulations of F-DOPA were subjected to quality controls before administration [29].

In our study the stable isotope [$^{19}$F]F-DOPA was used instead of [$^{18}$F]F-DOPA which is the fluorine radioisotope that decays by b+ emission; therefore the toxicity related to ionizing radiation was not investigated.

## MATERIALS AND METHODS

### Chemicals and solutions

All the experiments were accomplished using [19F]-(2*S*)-2-amino-3-(6-fluoro-3,4-dihydroxyphenyl) propanoic acid (F-DOPA), in which the fluorine atom present in the molecule is the stable isotope [19F]. F-DOPA and all other chemicals were purchased from Sigma-Aldrich (Milan, Italy) and used without any further purification.

Pre-formulation studies evaluating the chemical stability of F-DOPA were accomplished as previously described by Denora *et al*. (2017) using aqueous solutions at different pH values [29]. F-DOPA solubilized in phosphate buffer (0.05 M) at pH 8.0 and kept at 25 °C is unstable. Degradation products were formed, as evidenced by high pressure liquid chromatography (HPLC) analysis [29-31].

Based on the results obtained by F-DOPA pre-formulation studies, two different novel formulations, namely ND1 and ND2, were investigated [29]. Exactly weighted amounts of F-DOPA were solubilized in 1 ml of 1 mM lactate buffer aqueous solution (pH 5.0) in the presence of 1 mM $Na_2EDTA$ and 150 mM NaCl (300 mOsm/L) for

**Table 1.** Composition of the F-DOPA formulations.

| Formulation | Composition | | | |
|---|---|---|---|---|
| | **Buffer (mM)** | **$Na_2EDTA$ (mM)** | **pH** | **Ionic strength (mM)[a]** |
| **ND1[a]** | Lactate (1) | 1 | 5.0 | 150 |
| **ND2** | Lactate (1) | 1 | 5.0 | 0 |
| **ND3[b]** | Acetic acid (17) | 0 | 3.0 | 0 |

[a]The ionic strength was adjusted to 150 mM by the addition of NaCl. [b]Before its use the pH was adjusted by the addition of 100 μL/mL of a sterile solution of sodium bicarbonate (84 mg/mL).

ionic strength adjustment (ND1) or without 150 mM NaCl (ND2) (Table 1). The highest tested concentration of F-DOPA was 10 mg/ml. A 1 mM lactate buffer pH 5.0 was chosen to formulate the parenteral injectable solution as observed in lactated Ringer's solutions [32]. To slow-down the chemical reactions responsible for the instability of F-DOPA, chelating agents, such as EDTA, citric acid and tartaric acid, were used. In a previous study, the addition of $Na_2EDTA$ (0.15%) retarded the F-DOPA auto-oxidation reaction [2]. After preparation, the samples were kept in tightly closed glass vials of type I, under nitrogen and in the dark. On the basis of the *in vitro* and *in vivo* preliminary data, the ND2 was selected for further *in vivo* experiments.

In addition, the formulation ND3 was prepared by solubilizing the same active ingredient F-DOPA in an aqueous sterile solution of acetic acid (1.05 mg/ml), which had an acid pH ranging between 2.3 and 3.0. Immediately before injection, the pH of IASOdopa® was adjusted to 4.0-5.0 by the addition of a sterile solution of $NaHCO_3$ (8.4 g/100 ml). Each solution was injected within 30 minutes in *in vivo* experiments. The composition and use of the ND3 formulation mimic that of the commercially available IASOdopa® (IASON GmbH) and Dopacis® (CIS-bio international, IBA Molecular) [21-22].

### *In vivo* experiments: animal groups and dosing

The animals were divided into different experimental groups: Control untreated (CTRL U), control (CTRL), ND2 0.025 mg/kg, ND2 0.1 mg/kg, ND2 5 mg/kg in rats and 50 mg/kg in mice, and the ND2-vehicle (VD2); ND3 0.025 mg/kg, ND3 0.1 mg/kg, ND3 5 mg/kg in rats and 50 mg/kg in mice, and the ND3-vehicle (VD3). Despite the fact that the primary goal of this work is the evaluation of the tolerability of different F-DOPA formulations rather than animal dose translation to human, the highest doses of F-DOPA tested in mice of 50 mg/kg and rats of 5 mg/kg were 1785 and 178.6 folds, respectively, the human clinical dose which is 4 MBq/kg corresponding to 0.028-0.03 mg/kg of F-DOPA. The reported specific activity of the reference product IASOdopa® at the time of calibration was 30 GBq/mmol (0.3 GBq/ml of radiotracer per vial) [21]. The highest doses of F-DOPA tested in mice (50 mg/kg) and rats (5 mg/kg) based on body surface area (BSA) normalization correspond to the human doses of 4.05 mg/kg and 0.81 mg/kg, respectively [33]. Dose selection in toxicology study in animals is based on a safety factor of 1000 (rats or mice vs humans) as recommended for radiopharmaceuticals [34]. The lowest dose of F-DOPA of 0.025 mg/kg is in the range of the human clinical dose. It should be noted that the evaluation of plasma levels of F-DOPA metabolites to compare their AUC levels in animal and humans at the no observed adverse effects level was not performed in this work and therefore the safety factor used in this work is based on safety data and not on AUC data. Stock solutions of the formulations were prepared at the concentration of 10 mg/ml. Different concentrations of the ND2 and ND3 formulations were obtained by adding physiological solution immediately before the administration in order to avoid fast F-DOPA degradation. For each group, the solutions were injected as a bolus of maximal volume of 0.2 ml, which was adjusted on the basis of the body weight over a time period of 30 seconds, using a sterile plastic syringe equipped with a 27G needle for i.m., s.c., i.p. and i.v. administrations.

The control untreated (CTRL U) did not receive any treatment and was not sacrificed at the end of

the protocols. This group was monitored for possible treatment-related stress associated with the use of metabolic cage and the administration of physiological solution. The CTRL group received a sterile physiological solution.

The ND2 and ND3 0.025 mg/kg groups received 0.01 mg mass dose of F-DOPA, which is in the range of the human clinical dose of 1.75 mg for a man of 70 kg of body weight normalized to the rat body weight of 400 g and injected at the concentration of 0.05 mg/ml x injected volume.

The ND2 and ND3 0.1 mg/kg groups received 0.04 mg mass dose of F-DOPA injected at the concentration of 0.2 mg/ml x injected volume.

The ND2 and ND3 5 mg/kg in rats and ND2 and ND3 50 mg/kg in mice groups received 2 mg mass dose (10 mg/ml x injected volume) of F-DOPA. Rats and mice of the vehicle groups were treated with equal amounts of the formulations free of F-DOPA.

**Animals**

Male Wistar rats weighing 400 ± 33 g (Charles River Lab., Calco, Italy) (N° of total rats = 48) and male C57BL/6J mice weighing 44 ± 4 g (Charles River Lab., Calco, Italy) (N° of total mice = 19) were used. The experiments were focused on male mice and rats since no gender effects of F-DOPA are expected; additionally, no effects are expected on reproductive organs. After 1 week of observation, the rats and mice appeared homogeneous in terms of body weight and clinical behaviour as regards food and water consumption, urine and faeces production. The rats and mice were paired-housed (2 rats or mice per cage), with free access to food and water, and maintained at 18 ± 3 °C temperature and 68 ± 4 % of humidity.

**Ethics statement**

The protocol design was based on previously described principles and approved by competent authority [29]. In the single-dose extended-toxicity i.v. (SDET) study, a statistically significant evaluation of the endpoint was reached using a reduced number of animals and the sample size was calculated assuming a power of 0.85. In local tolerability experiments, in the absence of clinical reactions, the rats were not sacrificed and were used for further experiments after a period of washout, to keep their number to a minimum. The local tolerability experiments were performed in conscious rats and caused a moderate pain in the treated animals. The SDET i.v. study in rats and mice produced a mild-moderate pain in the treated animals. The i.v. treatments were performed under a mild anesthesia induced by i.p. injection of Zoletil 100 (20-30 mg/kg body weight). The blood sampling was performed under deep anesthesia produced by i.p. injection of Zoletil 100 (40 mg/kg body weight). The animals were sacrificed by overdose of Zoletil 100 (200 mg/kg body weight) followed by dislocation. The humane endpoints were: change in body weight ≥ 10%, change in the rectal body temperature ≥ 4 °C persistent for more than 3 days.

**Local tolerability tests in rats**

These experiments were performed in conscious rats (N° of rats = 24). In the case of F-DOPA, investigations with administration routes different from the main route used in humans are required [28, 34]. The i.m. tolerability experiments consisted in the administration of the formulations and relative vehicles into the tibialis anterior muscle area. The legs of the rats were shaved and the rats were divided into different experimental groups: controls (CTRL) (N° of rats = 3), ND2 (0.1 mg/kg) (N° of rats = 3), ND2 (5 mg/kg) (N° of rats = 3), ND3 (0.1 mg/kg) (N° of rats = 3) and ND3 (5 mg/kg) (N° of rats = 3). For each group, a volume of 0.2 ml of the drug solutions was injected into the left leg of the rat and the corresponding vehicle on the right leg. The control group received 0.2 ml of the physiological solution on the left leg and no treatment on right leg. The rats were monitored for skin local reactions such as redness, darkening and oedema formation within 24 h following the injection. Rats showing signs of adverse reactions following the i.m. injections were sacrificed. Rats not showing reactions entered the new protocols after 30 days of washout.

The same experimental design, animal groups and dosing were used for the evaluation of the animal reaction to the hindpaw injection (h.i.) of the formulations. The rats were monitored for hindlimb licking, shaking and withdrawal or oedema formation within 24 h following the h.i. injection.

i.m. and h.i. protocols:

| | | |
|---|---|---|
| CTRL | left / right | physiological solution<br>no treatment |
| ND2/ND3 | left / right | ND2 or ND3 formulations<br>VD2 or VD3 vehicles |

In the absence of adverse reactions, the same animals were used for s.c. and i.p. tolerability tests after 30 days of washout period. The animal groups and dosing were: control untreated (CTRL U), ND2 (5 mg/kg), VD2, control (CTRL), ND3 (5 mg/kg) and VD3.

These experiments were performed by observing the animal adverse reactions such as convulsion, piloerections or edema to i.p. and s.c. injections at the highest doses of the formulations and vehicles within 24 h from the administration.

s.c. and i.p. protocols:

CTRL
ND2 / ND3
VD2 / VD3

i.p. injection on abdominal area
s.c. injection on dorsal area

24h — clinical signs and reactions

Rats showing signs of adverse reactions were rapidly sacrificed to collect muscles and organs. The organs were placed in formaldehyde solution (10%) for histopathological analysis or stored at -80 °C for gene expression analysis. Cardiac blood and serum samples were conserved at -20 °C using appropriate anticoagulants.

**Single-dose extended-toxicity study in rats and mice**

According to the international guidelines for radiotracer development, a single-dose extended-toxicity study of intravenous administration of the F-DOPA formulations can be performed [34]. This is justified by the fact that the half-life of these drugs and their metabolites in humans and animals falls within the 12 hours from the administration.

The aim of this experiment was to evaluate the acute toxicity of a single dose of drugs and the reversibility of the observed effects after 14 days of follow-up in rats (N° of rats = 24) and mice (N° of mice = 16).

*Primary endpoint:* body weight changes.
*Secondary endpoints:* organs weight changes.

| i.v. protocol Experimental conditions | 0 | 1 | 13 days | 14 days |
|---|---|---|---|---|
| | ↓ | ↓ | ↓ | ↓ |
| | single dose administration | follow-up | metabolic cage | animal sacrifice |

The body weight and organ weight were evaluated in 24-h-fasted animals using digital analytical balances AV114C 500 g and 100 g, respectively, and data were stored for further analysis (OHAUS corp. NJ, USA).

The animal groups were: control untreated (CTRL U) (N° of rats = 3, N° of mice = 4), ND2 (0.025 mg/kg) (N° of rats = 3), ND2 (0.1 mg/kg) (N° of rats = 3), ND2 (5 mg/kg) (N° of rats = 3), ND2

(50 mg/kg) (N° of mice = 4), control (CTRL) (N° of rats = 3, N° of mice = 4), ND3 (0.025 mg/kg) (N° of rats = 3), ND3 (0.1 mg/kg) (N° of rats = 3), ND3 (5 mg/kg) (N° of rats = 3), and ND3 (50 mg/kg) (N° of mice = 4).

Each animal was weighed before treatment and anesthetized; then intravenous administration of the formulation under investigation was performed in the tail vein of the unconscious animals. After administration, the animals were placed in individual metabolic cages for not more than 2 days to reduce discomfort and distress and monitored for the following parameters: urine and feces production, consumption of water and food. At the end of the follow-up period, intracardiac blood samples were collected under deep anesthesia from unconscious living animals.

The biochemical parameters, plasmatic cytokines levels and oxidative stress were also investigated. Serum samples were conserved at -20 °C using appropriate anticoagulants for evaluating the toxicity of plasma biomarkers.

Fast-twitch glycolytic tibialis anterior (TA) and extensor digitorum longus (EDL) muscles, and the slow-twitch oxidative soleus (SOL) muscle, right and left kidney, heart, liver, lung and brain were removed from the animals sacrificed by cervical dislocation under deep anesthesia, wiped-off of liquid from the surface, weighted and stored at -80 °C for gene expression analysis. Hearth, skeletal muscles, liver and lung are expected to be target organs for both acetic acid and lactate vehicles used in the F-DOPA formulations under investigation, while brain can be considered a target organ for the F-DOPA. The effects of chemicals and drugs affecting metabolic pathways are muscle phenotype-dependent [35, 36]. Adrenal glands, that can also be considered a target tissue for the F-DOPA, were however not investigated due to the limited efficiency in the isolation of intact tissue and sampling in our lab. The kidney is the main organ involved in the elimination of the formulations under investigation. The adipose tissue was not accurately quantified in our experiments in rats and mice because it is located in different areas [37].

**Biochemical analysis of plasma markers**

The serum levels of cytokines such as IL-1b, IL-6 and TNF-alpha, as indices of inflammation, were evaluated using a Fluorescence-based Bioplex-Luminex multi-target technology (BIOCLARMA Srl, via M. Cristina, 121 - 10126 Torino – Italy). The serum cardiac troponin C, the aminotransferases (ALT and AST), the amylase, the creatine kinase (CK), the creatinine and urea used as indices of heart, liver, pancreatic, skeletal muscle and renal damages, respectively, were investigated by colorimetric and ELISA methodologies.

**Histological analysis of rat skeletal muscle section**

Atrophy, necrosis and vacuolar myopathy are observed in skeletal muscle following physio-pathological conditions or exposure to toxicants [38-42]. Histological analysis of skeletal muscle section allows to identify the mechanisms of muscle damages. Briefly, fixed muscle tissue specimens from muscles stained with hematoxylin and eosin were washed in water, dehydrated in ethanol, cleared in xylene, embedded in paraffin, and cut into serial sections of 10 μm thickness to determine the number of nuclei appearing as blue colored in the cytosolic compartment. The number of nuclei and capillaries of each fiber is normalized to the cross-sectional area (CSA) of the corresponding fiber. This staining did not distinguish myonuclei from other nucleus types (intra fiber nuclei, nuclei of the satellite cells and capillaries). The number of nuclei was obtained by counting all the nuclei around each individual fiber. Images from 10 random fields were acquired for the ten stained sections of each specimen using a D 4000 Leica DMLS microscope equipped with a camera and image analyzer (NIS elements-BR-Nikon). The cross-sectional area of muscle fibers was measured with QWin software (Leica).

**Isolation of total RNA, reverse transcription and real-time PCR**

All tissues for gene expression were prepared and data were analysed as previously described [43-46]. The mRNA expression of the genes was normalized to the best housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) selected among beta-actin (*β-Actin*), beta-2 microglobulin (*β-2m*) and *Gapdh* using Bestkeeper software (http://www.gene-quantification.de/bestkeeper.html). TaqMan Hydrolysis primer and probe (Life Technologies) for gene expression assays are reported in Table 2, except for *β-Actin* of mice, for which the assay was performed using the following sequences:

**Table 2.** RT-PCR gene primers.

| Gene | Protein | Id assay code |
|---|---|---|
| Rat | | |
| *Fbxo32* | Atrogin1 | Rn_00591730_m1 |
| *Casp3* | Caspase3 | Rn_0563902_m1 |
| *Casp9* | Caspase9 | Rn_00581212_m1 |
| *Trim63* | Murf_1 | Rn_00590197_m1 |
| *Map3k8* | Mitogen activated protein kinase kinase kinase 8 | Rn_01538561_m1 |
| *Bnip3* | Bcl2/adenoVD3rus e1b 19kda interacting protein | Rn_00821446_g1 |
| *Tnfa* | Tumor necrosis factor alpha | Rn_99999017_m1 |
| *Map1lc3a* | Lc3 | Rn_01536227_m1 |
| *Pargc1a* | Pgc1alpha | Rn_00580241_m1 |
| *NfKb1* | Nuclear factor of kappa light polypeptide gene enhancer in b cells | Rn_01399583_m1 |
| *Casp8* | Caspasi8 | Rn_00574069_m1 |
| *Trpv1* | Transient receptor potential cation channel, subfamily v, member 1 | Rn_00583117_m1 |
| *Cgrp* | Calcitonin gene related peptide | Rn01511353_g1 |
| Mice | | |
| *Bnip3* | Bcl2/adenoVD3rus e1b 19kda interacting protein | Mm_01275600_g1 |
| *Map1lc3a* | Lc3 | Mm_00458724_m1 |
| *Pargc1a* | Pgc1alpha | Mm_01208853_m1 |
| *Ucp1* | Uncoupling protein 1 | Mm_01244861_m1 |
| *Trim63* | Murf_1 | Mm_01185221_m1 |
| *Fbxo32* | Atrogin1 | Mm_00499523_m1 |
| *NfKb1* | Nuclear factor of kappa light polypeptide gene enhancer in b cells | Mm_00476361_m1 |
| *Tnfa* | Tumor necrosis factor alpha | Mm_99999068_m1 |
| *Casp3* | *Caspase3* | Mm01195085_m1 |
| *Casp9* | *Caspase9* | Mm00516563_m1 |

forward primer: 5′-CCAGATCATGTTTGAGAC CTTCAA-3′, reverse primer: 5′-CATACAGGGA CAGCACAGCCTY-3′ and Probe: VD3C-ACCC CAGCCATGTACGTA-MGB.

**Oxidative stress in mice**

The total antioxidant capacity of the serum samples from different mice was evaluated and expressed as Trolox equivalent in nmol /sample (μL) (Total Antioxidant Assay Kit, Sigma-Aldrich, MAK187). The malondialdehyde levels were evaluated in the serum sample of mice [Lipid Peroxidation (MDA) Assay Kit, Sigma-Aldrich, MAK085].

**Statistics**

The data are expressed as mean ± S.E.M. In *in vivo* experiments, changes in the wet weight of muscles and organs normalized to body weight were calculated as follows:

Organ weight (g) / body weight (g) after a follow-up period of 14 days in rats

Organ weight (g) / body weight (g) x 1000 after a follow-up period of 14 days in mice.

The percentage change of body weight was calculated as follows:

Percentage change of body weight (%) = [body weight (g) $t_{14}$ / body weight (g) $t_0$ x 100]-100

where the body weight t0 is the weight before the administration and the body weight t14 is the weight after a follow-up period of 14 days.

Data were exported and further analysed using Excel software (Microsoft Office 2010). Differences between

drug treatment groups were evaluated using one-way analysis of variance (ANOVA). Tukey's HSD test and Bonferroni correction were performed to counteract for multiple comparison and test for the significance of each individual hypothesis at a significance level of $\alpha = 0.05$. Student t-test, at $p < 0.05$ and $p < 0.001$ levels of significance, was used to test for significance between means.

In the single-dose extended-toxicity study in rats a sample size of 24 animals (N° = 3 rat/group) for 8 data groups was chosen based on the one-way ANOVA analysis assuming a 30% change in the body weight reduction following treatment with the F-DOPA formulations, the same standard deviation, an error of $\alpha = 0.05$ and a power of the study of 0.8. In mice experiments, a sample size of 16 animals (N° = 4 mice/group) for 4 data groups was chosen assuming a change of 30% in body weight reduction following treatment with the F-DOPA formulations, the same standard deviation, an error of $\alpha = 0.05$ and a power of the study of 0.89 (G*Power software 3.1.9.2, IDRE, UCLA USA).

## RESULTS

### *In vivo* experiments: local tolerability test in rat

A previous study has briefly reported local reaction of skeletal muscle following i.m. treatment of the animals with the ND3 formulation [29]. Here, we show that the i.m. injection of the ND3 dose 5 mg/kg (0.2 ml injected volume) into the left-hand tibialis anterior muscle of conscious rats (N° of rats per group =3) caused, for all treated animals, a local reaction characterized by marked redness and darkening of the injected area and a mild oedema formation within 30 minutes from the administration, clinically reversible after 4 h from the administration (Fig. 1A, B, C, D, E, F, G).

No clinically significant animal reactions to hind paw, i.p. and s.c. injections of the formulations were observed in the rats at the same dosing schedule (0.1 mg/kg-5 mg/kg) in terms of hindlimb licking, shaking and withdrawal, convulsion, piloerections and oedema formation within 24 h following injection.

Histological and RT-PCR investigations were performed in tibialis anterior muscle (muscle wet weight in controls = 520.5 ± 12 mg, N° muscles = 3) collected from rats after 30 minutes from the i.m. treatment with physiological solution (N° muscles/rats = 3/3), ND2 (5 mg/kg) (N° muscles/rats = 3/3) and ND3 (5 mg/kg) (N° muscles/rats = 3/3) formulations, VD2 (N° muscles/rats = 3/3) and VD3 (N° muscles/rats = 3/3). The histological analysis of tibialis anterior muscle sections revealed the presence of an elevated number of dead fibers (Number of sections per muscle = 10) in rats treated intramuscularly with the ND3 (5 mg/kg) and VD3 formulations which was significantly different *vs* controls, VD2 (5 mg/kg) and ND2 rat groups as determined by student t-test ($p < 0.05$) (Fig. 1E, 1F, 1G) (Table 3). In addition, this parameter was significantly elevated in ND3 *vs* VD3 vehicle ($p < 0.05$). The dead fibers of the ND3 group also showed a significantly reduced cross section area (CSA) as compared to that measured in the normal fibers ($p < 0.05$) (Table 3). Perimysium vacuoles were observed in the muscle sections from controls and ND2 and ND3 (Fig. 1E, 1F, 1G).

In preliminary RT-PCR experiments we had observed a potentiation of the apoptotic and autophagy genes [29]. Here we show that the mRNA levels of *Pgc1-alpha*, *Lc3*, *Cgrp*, *MapK3*, *Tnf-alpha* and *Caspase 3*, *8*, *9* genes were significantly enhanced after 30 minutes from the i.m. administration of 5 mg/kg ND3, as determined by one-way ANOVA ($F = 3.13$, $p < 0.05$) in the affected tibialis anterior muscles (Fig. 2) (N° muscles/rats = 5/5). This difference was determined for the 5 mg/kg ND3 group, as revealed by the Bonferroni and Tukey HSD tests (Tukey HSD test *p* values: ND3 *vs* CTRL, $p < 0.05$). The mRNA levels of *Atrogin1*, *Murf1*, *Bnip3* and *TrpV1* genes were not significantly affected. In addition, the mRNA levels of *Caspase 3*, *8*, *9*, *Lc3*, *Bnip3*, *Pgc1 alpha*, *Mapk3*, *Tnf-alpha* and *Cgrp* genes were enhanced following the VD3 treatment (Tukey HSD test *p* values: VD3 *vs* CTRL $p < 0.05$). The ND2 (10 mg/kg) treatment significantly enhanced the mRNA levels of the *Mapk3* and *Pgc1 alpha* genes ($p < 0.05$). No significant effect was observed in sampled genes with the VD2.

### *In vivo* experiments: single-dose extended-toxicity study in rats and mice

A single-dose extended-toxicity study was performed to evaluate the acute toxicity and the reversibility

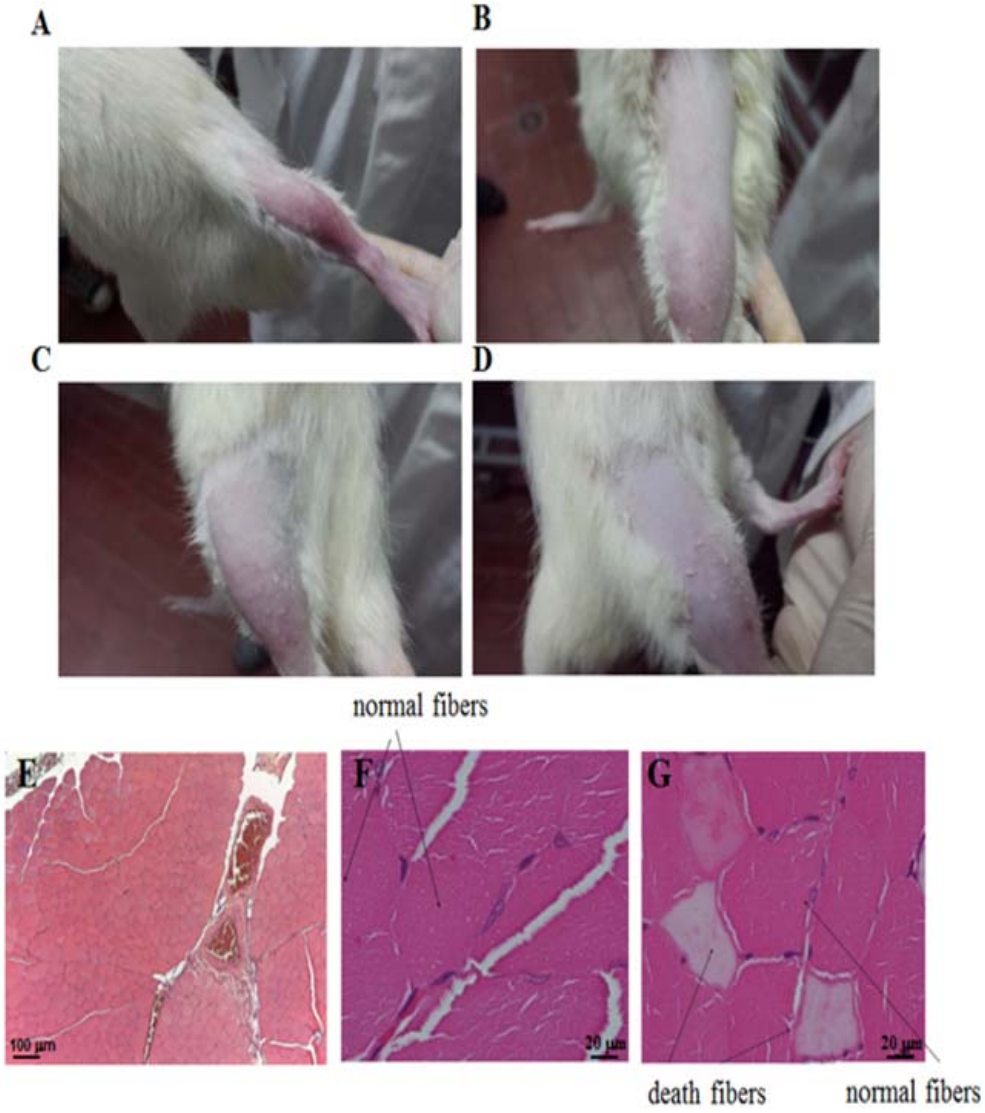


**Fig. 1.** Local reactions in tibialis anterior muscle after 30 min following i.m. administration of ND2, ND3 and their respective vehicles in male Wistar rats. (A) The i.m. injection of 0.2 ml of acetic acid-based ND3 formulation at the concentration of 10 mg/ml (5 mg/kg) caused a marked redness and darkening of the left-hand muscle. (B) No local reaction was observed after intramuscular injection of lactate/$Na_2$EDTA-based ND2 formulation (5 mg/kg). The administration of either acetic acid-based VD3 (C) or lactate/$Na_2$EDTA-based VD2 (D) did not cause visual local reaction in the right-hand muscles of the same rat. Representative sections of tibialis anterior (TA) muscle after 30 minutes from the local intramuscular injection into the same muscle are shown. (E) Control, physiological solution, (F) ND2 and (G) ND3. Perimisial vacuoles were observed in the muscle sections from controls, ND2 and ND3. Cytosolic opalescent areas, representative of dead fibers, were particularly observed in the ND3 muscle section. These fibers also showed a reduced cross-sectional area.

of the observed effects [28, 34] following a single i.v. dose in rats and a follow-up period of 14 days. At the end of the follow-up period, the animals were sacrificed and then all organs and blood were collected for further analysis. The body weight change analysis, performed by comparing the changes of body weight of the rats at the end of follow-up period *vs* the body weight before any treatment, revealed a mild but significant difference among groups as determined by one-way ANOVA

**Table 3.** Number of dead fibers and cross-sectional area (CSA) in tibialis anterior rat muscle.

| Treatments | Dead fibers | Normal fibers |
|---|---|---|
| VD3 vehicle<br>(N of sections per muscle =10)<br>(N of muscles = 3) | N of fibers = 57 ± 16$^{*,\#}$<br>CSA = 1620 ± 119 μm$^2$ | N of fibers = 47 ± 11*<br>CSA = 1930 ± 221 μm$^2$ |
| ND3 (5 mg/kg)<br>(N of sections per muscle =10)<br>(N of muscles = 3) | N of fibers = 97 ± 9 $^{*,\#,\$}$<br>CSA = 1610 ± 112 μm$^2$ | N of fibers = 23 ± 8*<br>CSA = 1970 ± 210 μm$^2$ |
| VD2 vehicle<br>(N of sections per muscle =10)<br>(N of muscles = 3) | N of fibers = 31 ± 7$^{\&}$<br>CSA = 1750 ± 210 μm$^2$ | N of fibers = 93 ± 13<br>CSA = 1930 ± 111 μm$^2$ |
| ND2 (5mg/kg)<br>(N of sections per muscle =10)<br>(N of muscles = 3) | N of fibers = 30 ± 8$^{\&}$<br>CSA = 1770 ± 201 μm$^2$ | N of fibers = 95 ± 9<br>CSA = 1970 ± 231 μm$^2$ |
| CTRL<br>(N of sections per muscle =10)<br>(N of muscles = 3) | N of fibers = 13 ± 3<br>CSA = 1770 ± 234 μm$^2$ | N of fibers = 101 ± 11<br>CSA = 2170 ± 230 μm$^2$ |

The histological analysis was performed after 30 min from the i.m. administration of F-DOPA formulations in male Wistar rats. The experimental groups were: CTRL, controls rats treated with physiological solution; ND3, rats treated with acetic acid-based F-DOPA (5 mg/kg); ND2, rats treated with lactate/$Na_2$EDTA-based F-DOPA (5 mg/kg); VD3, rats treated with the acetic acid vehicle solution; VD2, rats treated with lactate/$Na_2$EDTA vehicle solution. The data represent the mean ± S.D. of cross-sectional area (CSA) and number of fibers from randomly selected muscle sections of the same area. Data significantly different: $^{\$}$ND3 vs VD3 vehicle, *ND3 vs control (CTRL) treated with physiological solution, $^{\#}$ND3 vs VD2, $^{\&}$ND2 and VD2 groups vs CTRL ($p < 0.05$) as determined by student's *t* test.

($F = 5.37$, $p < 0.005$). This difference was determined in ND3 (5 mg/kg), as revealed by the Bonferroni and Tukey HSD tests [Tukey HSD test *p* values: ND3 *vs* CTRL, $p < 0.005$; ND3 (5 mg/kg) *vs* ND2 (5 mg/kg), $p < 0.05$; ND3 (5 mg/kg) *vs* VD3, $p < 0.0051$] (Fig. 3). No change in wet organ weight between groups (ANOVA one way, $F = 1.2$) was observed (Fig. 4). Moreover, the metabolic and physiological parameters of the rats, such as food and water consumption, urine and feces production were not significantly different among groups after 14 days of follow-up (ANOVA one way, $F = 1.1$) (Table 4).

The analysis carried out on intracardiac blood samples collected after 14 days of follow-up in rats did not reveal any significant changes, among experimental groups, in the serum levels of cardiac troponin C, aminotransferases ALT and AST, amylase, creatine kinase CK, creatinine and urea used as indices of heart, liver, pancreatic, skeletal muscle and renal damages (ANOVA one way, $F = 1.11$). No significant change in the serum TNF-alpha, IL-6 and IL-1b levels among groups was observed after treatments (ANOVA one way, $F = 1.1$) (Table 5).

RT-PCR experiments showed that the mRNA levels of *Pgc1-alpha* and *Cgrp* genes were significantly enhanced following i.v. ND3 (5 mg/kg) treatment and after 14 days of follow-up in rat brain as determined by one-way ANOVA ($F = 4.11$, $p < 0.05$), Bonferroni and Tukey HSD tests [Tukey HSD test *p* values: ND3 (5 mg/kg) *vs* CTRL, $p < 0.005$]. The *Cgrp* gene was also up-regulated in the VD3 group [Tukey HSD test *p* values: VD3 *vs* CTRL, $p < 0.005$] (Fig. 5). The expression level of *Atrogin-1*, *Murf-1*, *Bnip3*, *TrpV1*, *Caspase 3*, *8* and *9* genes were not significantly affected in brain (ANOVA one way, $F = 0.9$). The tibialis anterior muscle genes of the same rats were not significantly affected by the i.v. treatments (ANOVA one way, $F = 1.1$) (Fig. 5).

The effects of the higher doses of ND3 (50 mg/kg) and ND2 (50 mg/kg) formulations and their relative vehicles VD3 and VD2 were investigated

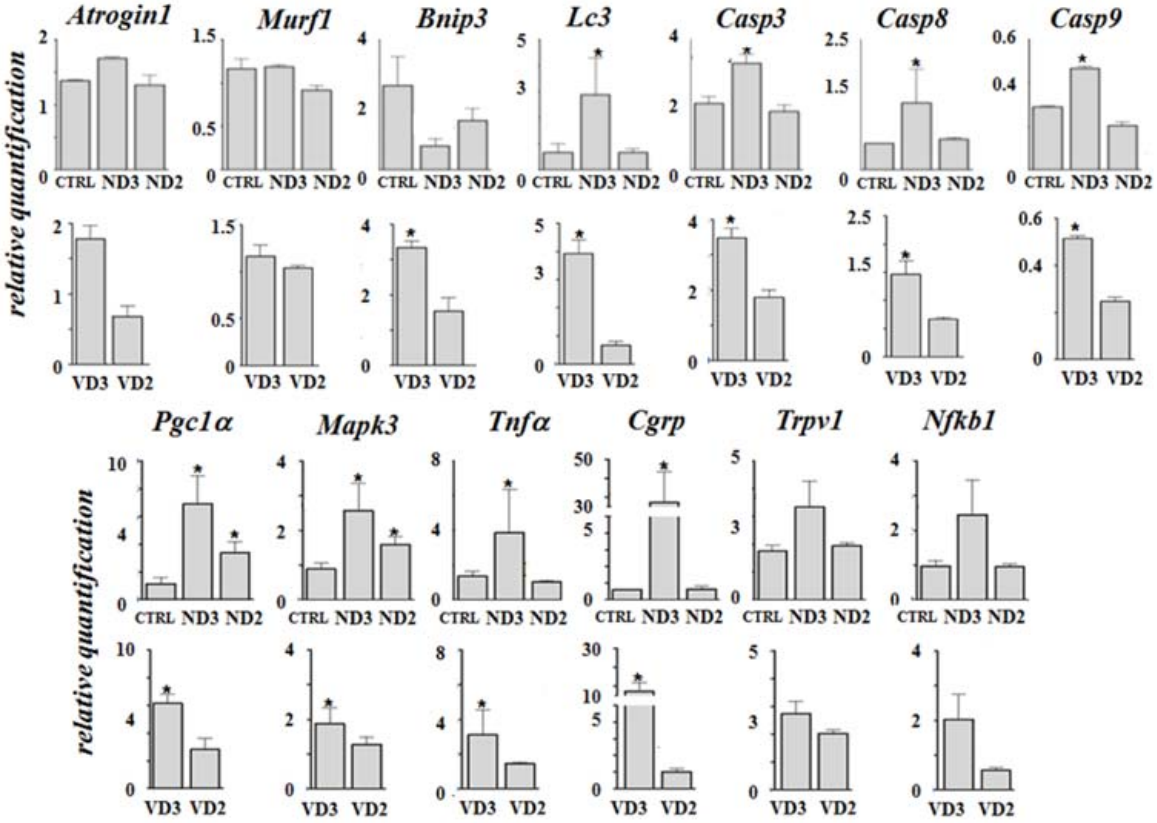


**Fig. 2.** Gene expression profile of tibialis anterior muscle after 30 min following i.m. administration of F-DOPA formulations and their respective vehicles in male Wistar rats. The experimental groups were: CTRL, controls rats treated with physiological solution; ND3, rats treated with acetic acid-based F-DOPA (5 mg/kg); ND2, rats treated with lactate/ $Na_2EDTA$-based F-DOPA (5 mg/kg); VD3, rats treated with the acetic acid vehicle solution; VD2, rats treated with lactate/$Na_2EDTA$ vehicle solution. Each bar represents the mean ± S.E.M. of three muscle samples. *One-way ANOVA analysis showed that the expression level of *Pgc1-alpha*, *Lc3*, *Cgrp*, *Casp3*, *8*, *9*, *Mapk3* and *Tnf-alpha* genes were significantly enhanced following treatments with ND3 vs CTRL controls ($p < 0.05$). *The *Casp3*, *8*, *9*, *Lc3*, *Bnip3*, *Pgc1-alpha*, *Mapk3*, *Tnf-alpha* and *Cgrp* genes were up-regulated following VD3 treatment vs CTRL ($p < 0.05$). The ND2 treatment significantly enhanced the expression levels of the *Mapk3* and *Pgc1-alpha* genes ($p < 0.05$).

in mice. ND3 (50 mg/kg) and VD3 treatments induced a significant loss in body weight after a period of follow-up of 14 days *vs* all groups (ANOVA $F = 5.22$, $p < 0.05$) (Fig. 6). This difference was determined in the ND3 and VD3 groups as revealed by the Bonferroni and Tukey HSD tests (ND3 *vs* CTRL, $p < 0.05$; ND3 *vs* ND2, $p < 0.020$; ND3 *vs* VD2, $p < 0.005$; VD3 *vs* CTRL $p < 0.020$; VD3 *vs* VD2, $p < 0.05$) (Fig. 6). No significant changes in body weight were observed following the ND2 (50 mg/kg) formulation and VD2 vehicle (ND2 *vs* CTRL, $p > 0.05$; VD2 *vs* CTRL, $p > 0.050$) administration.

No change in sampled wet organ weight between experimental groups (ANOVA one way, $F = 1$) was observed (Fig. 7). The metabolic parameters were not significantly different among groups after 14 days of follow-up in mice (ANOVA one way, $F = 0.94$) (Table 4).

The i.v. administration of ND3 dose 50 mg/kg and VD3 treatment significantly enhanced the *Lc3* and *Bnip3* gene expression of tibialis anterior muscle ($F = 3.2$, $p < 0.05$) as evaluated by the ANOVA one-way analysis in mice (Fig. 8). The *Lc3* gene was also significantly up-regulated following the ND2 treatment (50 mg/kg) ($F = 2.2$, $p < 0.05$). In contrast, the expression levels of *Atrogin-1*, *Murf-1*, *Pgc1 alpha*, *UCP1*, *Nfkb1*, *Caspase 3, 9* and *Tnf-alpha* genes were undetectable in tibialis anterior muscle after 14 days of follow-up even using

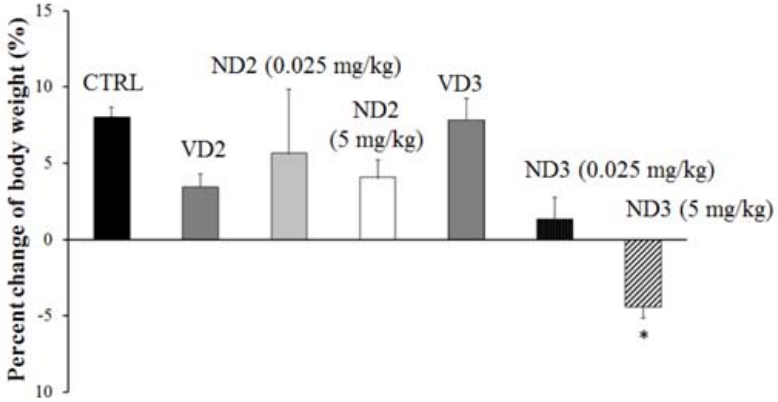


**Fig. 3.** Percent change in body weight (%) following i.v. administration of a single dose of F-DOPA formulations and a follow-up period of 14 days in male Wistar rats. The experimental groups were: CTRL, rats treated with physiological solution; VD2, rats treated with lactate/$Na_2$EDTA vehicle solution; ND2, rats treated with lactate/$Na_2$EDTA-based F-DOPA (0.025 mg/kg) and ND2 (5 mg/kg); VD3, rats treated with the acetic acid vehicle solution; ND3, rats treated with acetic acid-based F-DOPA (0.025 mg/kg) and ND3 (5 mg/kg). Each bar represents the mean ± S.E.M. of three animals. The statistical analysis performed with one-way ANOVA showed a significant difference among groups which was determined in the *ND3 (5 mg/kg) group that showed a mild drop in body weight vs controls ($p < 0.05$).

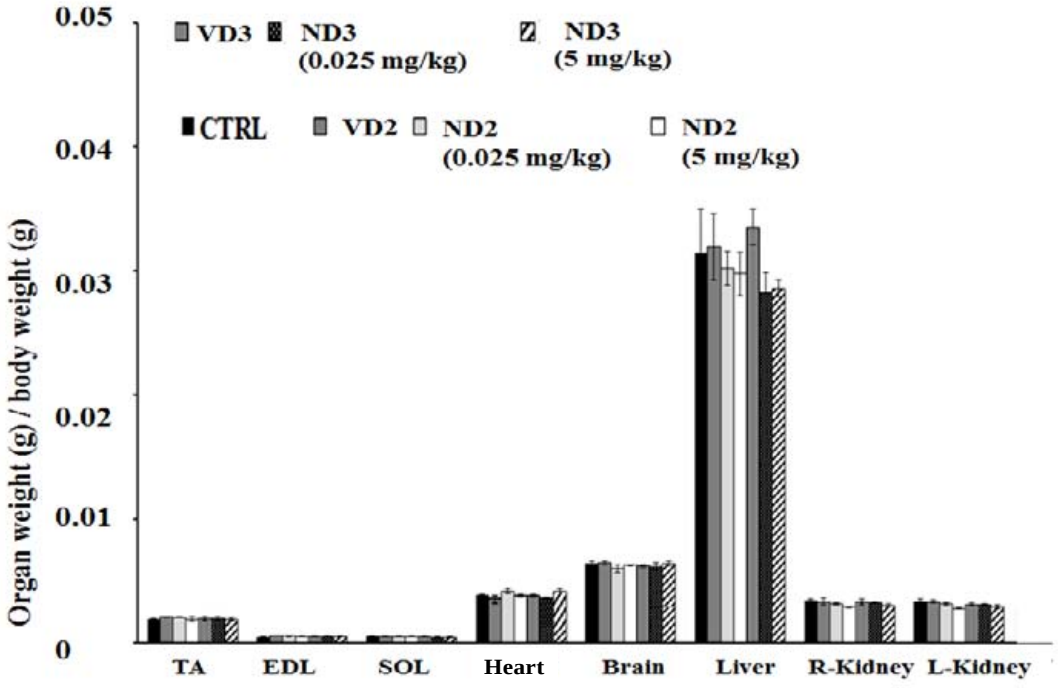


**Fig. 4.** Changes in organ wet weight normalized to body weight following i.v. administration of a single dose of F-DOPA formulations and a follow up period of 14 days in male Wistar rats. The experimental groups were: CTRL, rats treated with physiological solution; VD2, rats treated with lactate/Na2EDTA vehicle solution; ND2, rats treated with lactate/$Na_2$EDTA-based F-DOPA (0.025 mg/kg) and ND2 (5 mg/kg); VD3, rats treated with the acetic acid vehicle solution; ND3, rats treated with acetic acid-based F-DOPA (0.025 mg/kg) and ND3 (5 mg/kg). The organs were: tibialis anterior (TA), extensor digitorum longus (EDL) and soleus (SOL) muscles; the heart, brain, liver and kidneys (right and left). Each bar represents the mean ± S.E.M. of three samples. The statistical analysis performed with one-way ANOVA showed no significant difference among groups.

**Table 4.** Metabolic data following single i.v. dose administration of F-DOPA formulations and 14 days of follow-up in rats and mice.

| **Species Parameters** | **CTRL** | **VD2** | **ND2** (0.025 mg/kg in rat) | **ND2** (5 mg/kg in rat) (50 mg/kg in mice) | **VD3** | **ND3** (0.025 mg/kg in rat) | **ND3** (5 mg/kg in rat) (50 mg/kg in mice) |
|---|---|---|---|---|---|---|---|
| **Rats** **Food consumed (g)** | 24.67 ± 4.6 | 24.67 ± 4.8 | 20.33 ± 0.33 | 19.33 ± 1.3 | 27.33 ± 2.7 | 16.00 ± 3.5 | 15.33 ± 2.91 |
| **Water consumed (ml)** | 37.50 ± 12.5 | 23.67 ± 2.3 | 32.33 ± 7.9 | 31.67 ± 11.7 | 25.67 ± 6.9 | 24.33 ± 2.9 | 20.67 ± 5.20 |
| **Urine produced (ml)** | 16.33 ± 5.8 | 16.33 ± 0.9 | 14.00 ± 3 | 24.33 ± 10.6 | 17.00 ± 1 | 14.33 ± 0.7 | 14.33 ± 2.33 |
| **Feces produced (g)** | 12.67 ± 1.7 | 12.67 ± 3.5 | 9.00 ± 1.1 | 9.50 ± 0.9 | 9.00 ± 0.7 | 6.00 ± 2 | 7.00 ± 0.66 |
| **Mice** **Food consumed (g)** | 2.15 ± 0.6 | 1.44 ± 0.7 | - | 2.00 ± 1.1 | 1.54 ± 0.75 | - | 1.42 ± 1.30 |
| **Water consumed (ml)** | 3.33 ± 0.9 | 10.67 ± 2.3 | - | 10.67 ± 2.3 | 7.33 ± 1.45 | - | 9 ± 4 |
| **Urine produced (ml)** | 1.65 ± 0.2 | 2.54 ± 0.2 | - | 2.00 ± 0.6 | 1.56 ± 0.77 | - | 2.17±0.4 |
| **Feces produced (g)** | 0.75 ± 0.1 | 0.65 ± 0.2 | - | 0.93 ± 0.3 | 0.83 ± 0.29 | - | 0.655±0.41 |

The data were not significantly different among groups as determined by one-way analysis of variance. The data are the means ± SEM of 3 animals.

pre-amplification methodology for total mRNA extraction, suggesting a marked nucleotide down-regulation. Brain gene expression was not affected following treatments in mice (ANOVA one way, F = 0.92) (Fig. 8). The mRNA content was abnormally reduced in the muscle and the brain of ND3 (50 mg/kg) and VD3-treated mouse *vs* controls (Table 6).

Oxidative stress analysis in serum samples following a single dose i.v. administration of F-DOPA formulations and a follow-up period of 14 days revealed no significant changes in the total antioxidant capacity of the serum samples from different animals, expressed as Trolox equivalent in nmol/sample (μL) (Total Antioxidant Assay Kit, Sigma-Aldrich, MAK187) and MDA levels in mice (ANOVA one way, F = 0.81) [Lipid Peroxidation (MDA) Assay Kit, Sigma-Aldrich, MAK085].

## DISCUSSION

In the present work, the toxicity profile of novel cold F-DOPA formulations was investigated to overcome the chemical instability and poor tolerability of the currently known and chemically used formulations. These formulations have the disadvantage that, immediately before injection, their pH has to be adjusted to 4.0-5.0 by the addition of a sterile aqueous solution of sodium bicarbonate (84 mg/mL). Furthermore, undesirable pain at the injection site has been reported in rare cases, which resolve within minutes without corrective measures [21, 22, 26, 27]. To overcome these drawbacks, we developed new preparations through pre-formulation and formulation studies, which could guarantee the chemical stability of F-DOPA in solution at pH values compatible with the direct infusion, also avoiding or minimizing acute adverse reactions.

The novel formulations under investigation were based on a pH buffering component capable of reducing the catalyzing actions of cations on F-DOPA degradation in the presence (ND1 formulation) or absence of NaCl (ND2 formulation) as osmotic component. Furthermore, an acetic acid-based F-DOPA (ND3) formulation mimicking that commercially available i.e. IasoDOPA® was investigated.

In our previous *in vitro* experiments, the lactate $Na_2$EDTA-based F-DOPA ND2 formulation had been less cytotoxic than the lactate $Na_2$EDTA-based F-DOPA with added NaCl (ND1), and the acetic acid-based ND3 formulations in different cell types [29].

We found that the i.m. injection of ND3 dose 10 mg/kg caused a local muscular reaction within 30 minutes from the administration, characterized by

**Table 5.** Cytokine and biological marker levels following single i.v. dose administration of F-DOPA formulations and 14 days of follow-up in rats.

| | **IL-1b (pg/mL)** | **IL-6 (pg/mL)** | **TNF-α (pg/mL)** | **Cardiac Troponin I (ng/ml)** | **ALT (mg/dl)** | **AMILASI (mg/dl)** | **AST (mg/dl)** | **CK (mg/dl)** | **CREA (mg/dl)** | **UREA (mg/dl)** |
|---|---|---|---|---|---|---|---|---|---|---|
| VD2 (N samples =3) | 27.5 ± 1 | OOR < | OOR < | 2.7 ± 0.5 | 42.0 ± 1.63 | 1205.0 ± 216.4 | 103.5 ± 7 | 785.0 ± 174 | 0.4 ± 0.01 | 21.5 ± 9.4 |
| ND2 (N samples =3) (0.025 mg/kg) | 39.49 ± 4 | OOR< | OOR < | 0.784 ± 0.02 | 54 ± 3 | 1350 ± 120 | 122 ± 19 | 1130 ± 123 | 0.36 ± 0.03 | 35 ± 5 |
| ND2 (N samples =3) (5 mg/kg) | 30.8 ± 3 | OOR< | OOR < | 6.3 ± 0.9 | 51.7 ± 7 | 1223.3 ± 78.9 | 172.0 ± 19 | 1080.0 ± 170 | 0.5 ± 0.06 | 24.7 ± 2.4 |
| VD3 (N samples =3) | 54.5 ± 15 | OOR< | OOR < | 5.8 ± 1.8 | 66.3 ± 11 | 1526.7 ± 211 | 144.7 ± 14 | 2220.0 ± 630 | 0.4 ± 0.04 | 35.7 ± 1.2 |
| ND3 (N samples =3) (0.025 mg/kg) | 37.0 ± 8 | OOR < | OOR < | 12.1 ± 5.17 | 52.3 ± 3.53 | 1243.3 ± 77.5 | 146.7 ± 14.5 | 1386.7 ± 402.9 | 0.4 ± 0.03 | 33.3 ± 1.2 |
| ND3 (N samples =3) (5 mg/kg) | 139.7 ± 71 | OOR < | OOR < | 8.1 ± 3.12 | 211.0 ± 73 | 1076.7 ± 167.4 | 297.3 ± 87 | 1550.0 ± 274 | 0.2 ± 0.05 | 35.0 ± 6.1 |
| CTRL (N samples =3) treated with physiological solution | 23.8 ± 8 | OOR < | OOR < | 10.9 ± 4.6 | 58.7 ± 6.7 | 1520.0 ± 38 | 132.7 ± 17 | 1990.0 ± 300 | 0.3 ± 0.03 | 31.3 ± 1.2 |

The data were not significantly different among groups as determined by one-way analysis of variance. OOR (out of range).

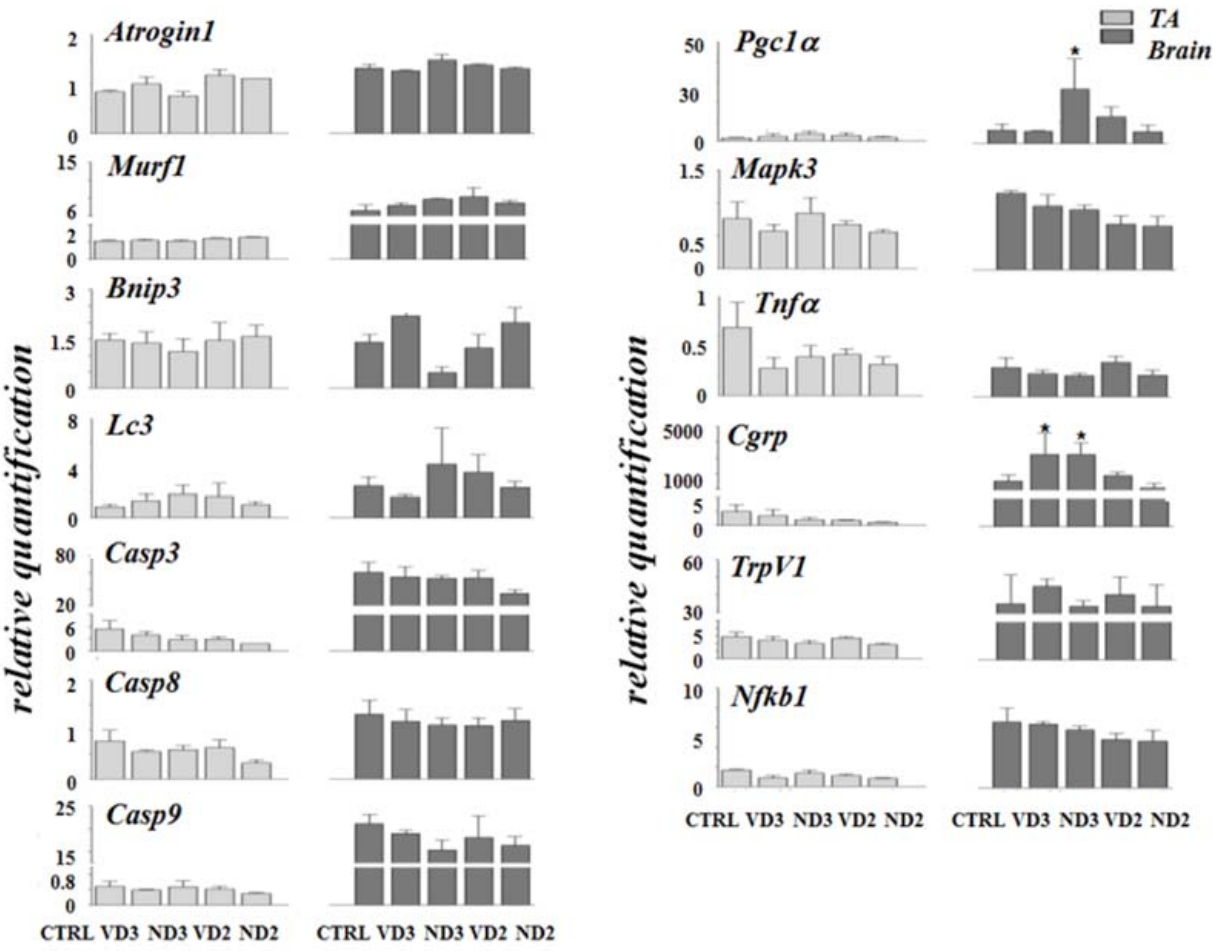


**Fig. 5.** Gene expression profiles of whole brain and tibialis anterior (TA) muscle following i.v. administration of a single dose of F-DOPA formulations and their respective vehicles and a follow-up period of 14 days in male Wistar rats. The experimental groups from left to right: CTRL, control rats treated with physiological solution; VD3, rats treated with the acetic acid-based vehicle solution; ND3, rats treated with acetic acid-based F-DOPA (5 mg/kg); VD2, rats treated with lactate/$Na_2$EDTA-based vehicle; ND2, rats treated with lactate/$Na_2$EDTA-based F-DOPA (5 mg/kg). Each bar represents the mean ± S.E.M. of three muscle and brain samples. One-way ANOVA analysis showed that the expression level of *Pgc1-alpha* and *Cgrp* genes was significantly enhanced following ND3 treatment and a follow-up period of 14 days in rat brain vs controls ($p < 0.05$). The **Cgrp* gene was also upregulated in the VD3 vehicle group vs controls ($p < 0.05$). No changes in the gene expression following i.v. treatment with the F-DOPA formulations were observed in the muscles of the same rats.

redness of the area, which was not observed with the VD3, ND2 and VD2 or with physiological solution treatments. These findings suggest that this reaction is associated with rapid F-DOPA degradation into the muscle compartment, specifically observed with the acetic acid-based ND3 formulation. A loss of tibialis anterior muscle fibers, with reduced CSA and a mild atrophy, was observed in rat muscle treated with ND3 and in part also with the acetic acid-based vehicle VD3. The fiber loss and atrophy are associated with the induction of the apoptotic *Caspase 3*, *8* and *9* genes as well as of the *Tnf-α* gene, suggesting that these pathways can be responsible for the observed local effects of the acetic acid-based F-DOPA formulation ND3 and its vehicle VD3. *Caspase 8* is mostly involved in receptor-mediated apoptosis, whereas *Caspase 9* is involved in the mitochondria apoptotic pathway [47]. Our data are in line with the well-known cytotoxicity of acetic acid in different cell types. The acetic

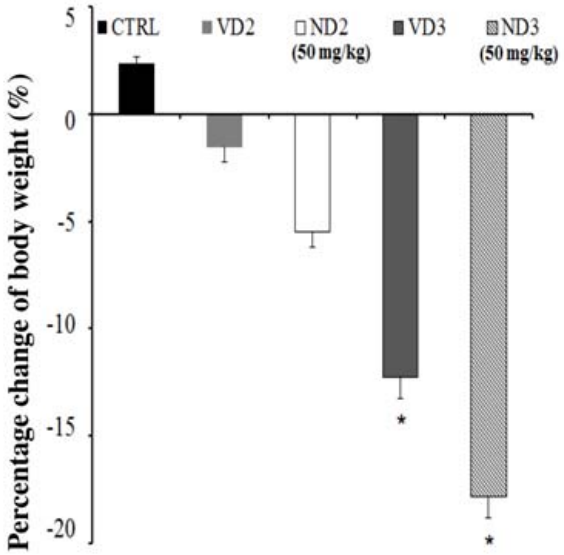


**Fig. 6.** Percentage change in body weight (%) following i.v. administration of a single dose of F-DOPA formulations and a follow-up period of 14 days in male C57BL/6J mice. The experimental groups were: CTRL, mice treated with physiological solution; VD2, mice treated with lactate/$Na_2$EDTA vehicle, ND2, mice treated with lactate/$Na_2$EDTA-based F-DOPA (50 mg/kg); VD3, mice treated with the acetic acid-based vehicle solution; ND3, mice treated with acetic acid-based F-DOPA (50 mg/kg). Each bar represents the mean ± S.E.M. of four animals. The statistical analysis performed with one-way ANOVA showed a significant difference among groups. This difference was determined in the *ND3 group and VD3 group that showed reduced body weight after 14 days of follow-up vs controls ($p < 0.05$).

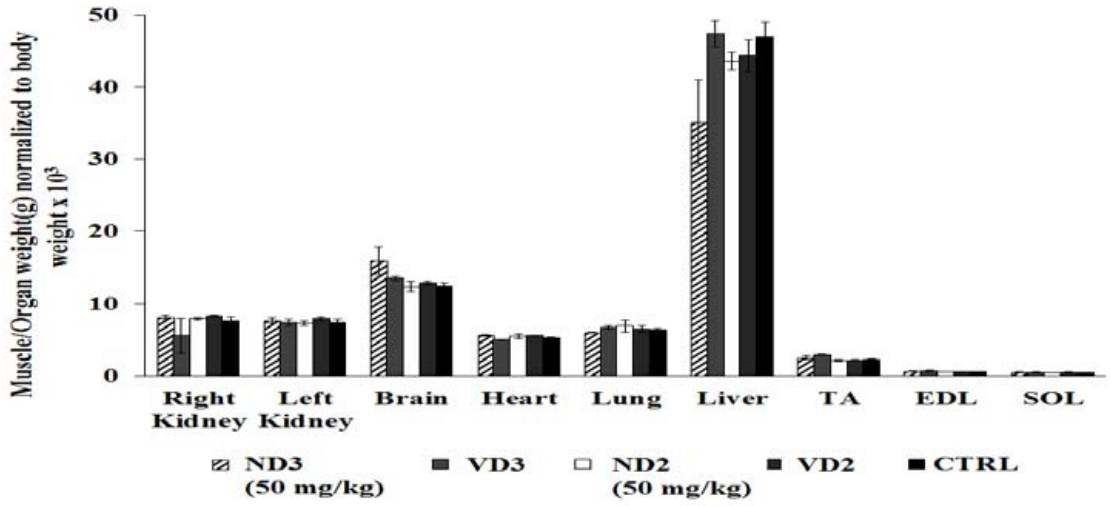


**Fig. 7.** Change in organ wet weight normalized to body weight following i.v. administration of a single dose of F-DOPA formulations and a follow-up period of 14 days in male C57BL/6J mice. The experimental groups were: CTRL, mice treated with physiological solution; VD2, mice treated with lactate/$Na_2$EDTA vehicle, ND2, mice treated with lactate/$Na_2$EDTA-based F-DOPA (50 mg/kg); VD3, mice treated with the acetic acid-based vehicle solution; ND3, mice treated with acetic acid based F-DOPA (50 mg/kg). The organs were: tibialis anterior (TA), extensor digitorum longus (EDL) and soleus (SOL) muscles; the heart, brain, lung, liver and kidneys (right and left). Each bar represents the mean ± S.E.M. of four samples. The statistical analysis performed with one-way ANOVA showed no significant difference among groups after 14 days of follow-up.

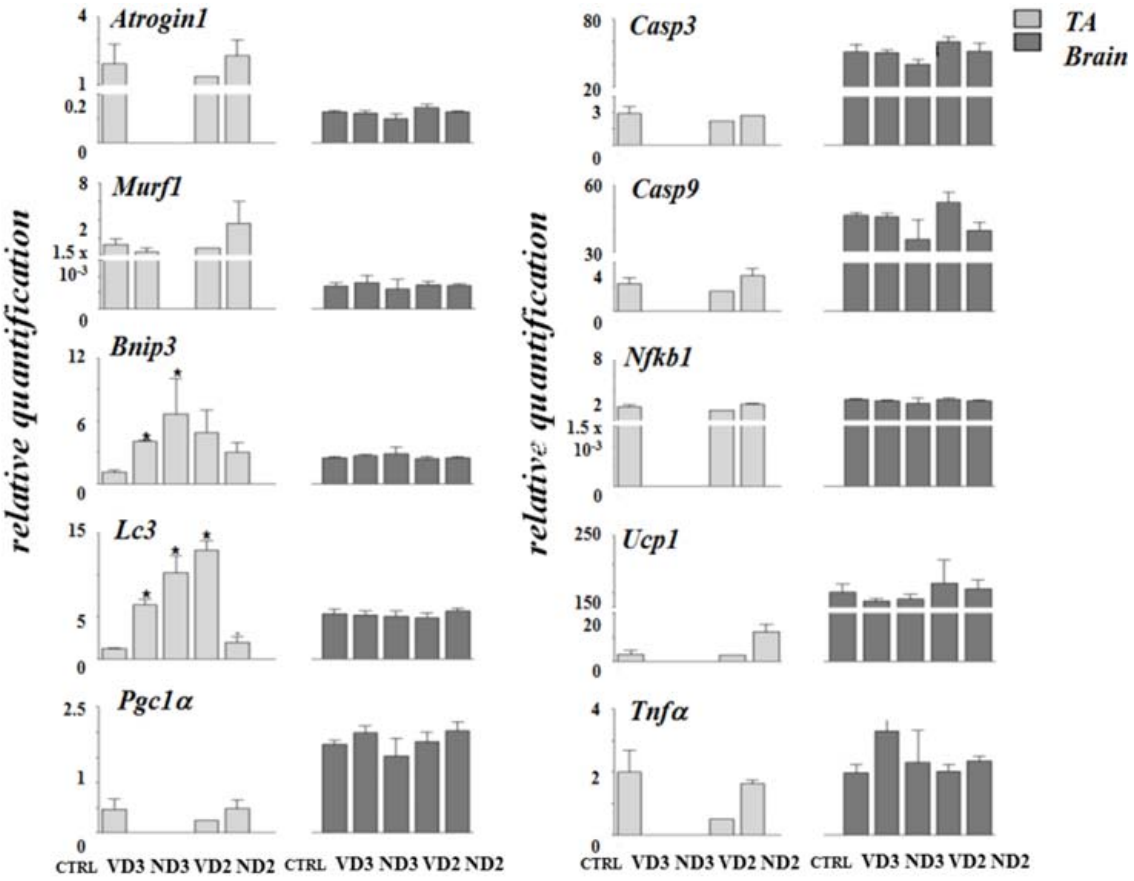


**Fig. 8.** Gene expression profile of whole brain and tibialis anterior (TA) muscle following i.v. administration of a single dose of F-DOPA formulations and a follow-up period of 14 days in male C57BL/6J mice. The experimental groups from left to right: CTRL, control mice treated with physiological solution; VD3, mice treated with the acetic acid-based vehicle solution; ND3, mice treated with acetic acid-based F-DOPA (50 mg/kg); VD2, mice treated with lactate/$Na_2$EDTA-based vehicle; ND2, mice treated with lactate/$Na_2$EDTA-based F-DOPA (50 mg/kg). Each bar represents the mean ± S.E.M. of at least four muscle and brain samples. The **Lc3* and *Bnip3* genes were up-regulated following ND3 (50 mg/kg) and VD3 treatments in tibialis anterior muscle (One-way ANOVA $p < 0.05$). The **Lc3* gene was also significantly up-regulated following the ND2 (50 mg/kg) treatment vs controls ($p < 0.05$). In contrast, the expression levels of *Atrogin-1*, *Murf-1*, *Pgc1 alpha*, *UCP1*, *Nfkb1*, *Casp3*, *9* and *Tnf alpha* genes were undetectable in tibialis anterior muscle.

acid-induced apoptotic cell death is a well-studied phenomenon in yeasts, which is characterized by early reactive oxygen species (ROS) accumulation and by the release of cytochrome c from mitochondria (cyt c) accompanied by respiratory chain defects [48]. There is a strong down-regulation of the enzymes involved in glycolysis/gluconeogenesis, up-regulation of pentose cycle and down-regulation of adenosine kinase enzyme involved in the nucleotide metabolism [48, 49].

In the same muscles treated with ND3 (5 mg/kg) and its vehicle VD3, an up-regulation of the autophagic *Lc3* and *Bnip3* genes was found; these genes may have dual actions contributing to the fiber damage observed in our experiments or exerting cytoprotection against insults. *Lc3* gene is indeed involved in the autophagosome formation, whereas *Bnip3* gene is involved in the mitophagy mechanism [50, 51]. The induction of apoptosis was, indeed, observed in the muscles treated with ND3 and VD3, however, in the absence of the expected induction of proteolytic effectors *Atrogin-1* and *Murf1* genes, in the absence of *NfκB* gene induction and in the absence of the muscle wet weight loss suggesting no significant proteolysis *in vivo*. These apparent

**Table 6.** mRNA content of tibialis anterior muscle and brain in mice.

| Treatments | Tibialis Anterioris muscle mRNA (ng/mL) | Brain mRNA (ng/mL) |
|---|---|---|
| CTRL (N muscles/mice = 4/4) | 221 ± 12 | 7498 ± 280 |
| ND3 (50 mg/kg) (N muscles/ mice = 4/4) | 46 ± 7* | 3916 ± 100* |
| VD3 (N muscles/mice = 4/4) | 34 ± 3* | 4547 ± 270* |
| ND2 (50 mg/kg) (N muscles/ mice = 4/4) | 187 ± 14 | 8055 ± 460 |
| VD2 (N muscles/ mice = 4/4) | 195 ± 10 | 5943 ± 383 |

The total mRNA content was evaluated following i.v. administration of a single dose of F-DOPA formulations and a follow-up period of 14 days in male C57BL/6J mice. The experimental groups were: CTRL, control mice treated with physiological solution; ND3, mice treated with acetic acid-based F-DOPA (50 mg/kg); ND2, mice treated with lactate/$Na_2$EDTA-based F-DOPA (50 mg/kg); VD3, mice treated with the acetic acid vehicle solution; VD2, mice treated with lactate/$Na_2$EDTA vehicle solution. The data represents the mean ± S.D. of mRNA content of *tibialis anterior* muscle and brain. Data significantly different: *ND3 and VD3 vs control (CTRL) treated with physiological solution, ($p < 0.05$) as determined by student's *t* test.

discrepancies can be explained by the induction of a mechanism counterbalancing the observed up-regulation of the apoptotic and inflammatory genes. In this context, the observed up-regulation of the mitochondria master gene *Pgc1a* involved in the mitochondrial biogenesis in the treated muscle may have counterbalanced the expected loss of mass following acetic acid-based F-DOPA formulation ND3 treatment. This gene was also up-regulated in the ND2 (5 mg/kg)-treated muscles. The *Atrogin1/ FoxO* activity is reported to be modulated by *Pgc1α*, a critical cofactor for mitochondrial biogenesis [50-53]. High levels of *Pgc1α* during catabolic conditions serve to protect muscle mass as observed in those conditions associated with an abnormal expression of *FoxO3* [53-56]. The trophic action on muscle mass of *Pgc1α* cofactor is dependent on the modulation of autophagy-lysosome and ubiquitin-proteasome signaling. Then, the observed up-regulation of the autophagy genes can be a *Pgc1α*-mediated protective mechanism eliminating damaged organelles or mitochondria. *Pgc1α* inhibits *FoxO3* and *NFκB* gene and reduces protein degradation without affecting protein synthesis. These cofactors prevent the abnormal activation of proteolytic pathways.

Although we have not observed any sign of pain reactions following local i.m., s.c., i.p and h.i. injections in all treated groups, some inflammatory and pain genes such as *Mapk3* and *Cgrp* but not *Trpv1* were up-regulated in the ND3 (5 mg/kg) and VD3-treated muscles and *Mapk3* was also up-regulated in the ND2 (5 mg/kg)-treated muscles suggesting a stimulation of pain sensory pathways [57, 58]. Other than the acetic acid, the F-DOPA degradation products may play a role in pain. Indeed, the dopamine system is associated with pain perception [59, 60].

A single i.v. dose of ND3 (5 mg/kg) caused mild symptoms in rats such as a reduction in body weight with no change in the sampled organ weight, metabolic parameters and serum markers, after 14 days of follow-up, suggesting no effects of ND3, VD3, ND2 and VD2 on the organ functionality and animal physiology. Brain *Pgc1α* and *Cgrp* were the only up-regulated genes in the ND3 (5 mg/kg) i.v.-treated rats.

The i.v. treatment of the mice with 50 mg/kg of ND3 and relative vehicle VD3 caused a loss in body weight of -17.8 ± 8% and -12.6 ± 6% *vs* controls, respectively, after a period of 14 days of follow-up. This effect was observed in the absence of changes in sampled organs weight and metabolic parameters, but the involvement of other tissues such as the adipose tissues cannot be excluded.

It was found that the autophagy genes *Lc3* and *Bnip3* were up-regulated in the muscles from the i.v.

ND3 (50 mg/kg) and VD3-treated mice. *Lc3* was also up-regulated in the muscles from ND2 (50 mg/kg)-treated mice. In contrast, the apoptotic, inflammatory and pain genes, and the *Ucp1* and *Pgc1α* genes were all down-regulated following the ND3 and VD3 treatment. These findings appear to be related to the loss of nucleotide content, as demonstrated by the fact that in our experiments the mRNA content was significantly reduced following treatments with ND3 and VD3 *vs* controls. Other than apoptotic action, acetic acid could indeed affect nucleotide synthesis in yeast, leading to a down-regulation of adenosine kinase enzyme that catalyzes ATP-dependent phosphorylation of purine nucleosides to monophosphate derivatives [48, 49].

It should be stressed that the highest dose of the acetic acid vehicle (VD3) formulation tested in our experiments, which was 0.51 mg/kg i.v., is much lower than the reported intravenous LD50 of acetic acid in mice, which is 525 mg/kg i.v. [61]. The use of lactate as vehicle is also favoured by the fact that lactated Ringer's solution was reported to reduce inflamatory diseases in patients with acute pancreatitis [62].

Despite most of the observed *in vivo* long-term effects were also related to the acetic acid action, the F-DOPA degradation may contribute to the short-term effects observed in previous *in vitro* experiments and in muscles following the i.m. administration [29]. The observed up-regulation of *Lc3* and other related genes such as *Bnip3 and Pgc1α* that we observed following 14 days of follow-up in mice muscle can be related with the long-term cytoprotective action exerted by these genes against acetic acid-related insults. While the F-DOPA effects are lost within 12-24 hours following the administration, as expected taking into account the short half-life of the drug which is 12 hours, the metabolic acetic acid effects appear to be persistent, affecting the muscle metabolism in the long term.

## CONCLUSION

Two novel [19F]F-L-DOPA beta-cyclodextrin (CD)-based formulations have also been investigated in our labs [63]. The chemical stability of these formulations was found to be longer than IASOdopa® with the addition of thiol-antioxidant agents, in particular L-Cysteine. *In vitro* experiments investigating on the effects of these formulations on different cell types showed that the [19F]F-L-DOPA beta-cyclodextrin (CD)-based formulations were less cytotoxic compared to F-DOPA solutions; these were also better tolerated than the IASOdopa® formulation in *in vivo* tolerability tests on adult male rat, showing no significant effects on body weight and on their dried organs weight. Increase in the serum CK and AST was occasionally observed with these formulations following the i.v. administration to the rats, which has been attributed to the effect of the vehicle alone that significantly enhanced the serum levels of these markers [63]. The F-DOPA lactate-based formulations have been developed using pharmaceutical excipients which can increase the chemical stability and solubility of the active ingredient and allow the direct i.v. administration without affecting its intrinsic water solubility. On the contrary, F-DOPA beta-cyclodextrin formulations are based on the use of cyclodextrin, which are known excipients used to increase the water drug solubility but do not always improve long-term stability of the active ingredient. For these reasons, despite the [19F]F-L-DOPA beta-cyclodextrin-based formulation represents an innovation for diagnostic purposes the current profile of the lactate-$Na_2$EDTA based F-DOPA formulation appears to be superior in *in vitro* experiments on cell lines and in *in vivo* animal experiments.

ND2 did not affect body weight or serum marker levels, but the muscle and brain gene expressions were mildly but not significantly affected in rats and mice, suggesting that the lactate-based formulation is better tolerated than the commercially available and clinically used acetic acid-based formulations. The ND2 formulation is also superior than the [19F]F-L-DOPA beta-cyclodextrin (CD)-based formulations in terms of tolerability and solubility [63].

One limitation of our study is that the toxicity related to ionizing radiation was not investigated in the present work because the stable isotope [$^{19}$F]F-DOPA was used instead of [$^{18}$F]F-DOPA which is the fluorine radioisotope that decays by b+ emission. In addition, pharmacokinetic investigation of the novel formulations was also not performed. Further experiments are needed to evaluate the efficacy of these novel formulations in human setting.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experimental protocols were submitted on 29 October 2013 and approved by the competent authorities C.E.S.A. of University of Bari and Italian Health Department of Rome (Art. 9 del D.L. 116/92, N° 33/2000-B del Dip. degli Alimenti e Nutrizione e della Sanità Pubblica).

## CONSENT TO PUBLISH

Not applicable.

## AVAILABILITY OF DATA AND MATERIALS

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request, but not publicly available.

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## AUTHORS' CONTRIBUTIONS

D. T., A. S., N. D., and M. F. participated in research design; F. M., G. C., A. M., N. Z., A. C., A. L., V. L., and A. C. conducted the experiments; V. D., and A. T. contributed new reagents or analytical tools; F. M., G. C., N. Z., A. C., and A. L. performed data analysis; D. T., A.S., N. D. and R.S. wrote or contributed to the writing of the manuscript. All authors approve the final version of the manuscript.

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## CONFLICT OF INTEREST STATEMENT

All authors have read the journal's policy on disclosure of potential conflicts of interest and have disclosed any financial or personal relationship with organizations that could potentially be perceived as influencing the described research.

## ABBREVIATIONS

[$^{18}$F]F-DOPA, 6-[18F]fluoro-L-dihydroxyphenylalanine; PET, positron emission tomography; SPECT, single photon emission computed tomography; NET, neuroendocrine tumors; ND1, lactate/NaCl/$Na_2$EDTA/F-DOPA; ND2, lactate/ $Na_2$EDTA/F-DOPA; ND3, acetic acid/F-DOPA; SDET, Single-Dose Extended-Toxicity; CSA, cross sectional area.

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