

TARGETING HIGH DOSE NIFEDIPINE-INDUCED HYPERTROPHY IN TISSUES WITH HYDROCORTISONE-LOADED NANOCARRIERS

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Abstract

We aim to study organ penetrability and anti-inflammatory effect of Hydrocortisone-loaded nanoparticles (HC-nano) after inducing hypertrophy with previous very-high dose medium-term Nifedipine administration. **Materials and methods:** Experiment was conducted in two phases on 60 adult, healthy Wistar rats, randomly divided initially in two groups (Phase 1), respecting 1:1 ratio between males and non-pregnant females, within each group. Group A1 (control) included 20 rats (10M/10F) and received no medication. Group B1 included 40 rats (20M/20F) and received oral Nifedipine 100 mg/kg body weight daily (approximately 100x therapeutic human dose), for 4 weeks. When Phase 1 ended, 8 rats did not survive in Group B1, and 40% (approximated to nearest integer) of rats of each sex, within each group were sacrificed, organs were harvested and analyzed microscopically. The rest of living rats entered Phase 2: Group A2 (control) remained with 12 rats (6M/6F) continuing with no medication; Group B2 counted 18 rats (10M/8F) that were given oral HC-nano 2 mg/kg body weight daily (therapeutic dose), for another 4 weeks. After Phase 2 completion, all animals were sacrificed, and tissues were microscopically analyzed. **Results:** Nifedipine induced statistically significant hypertrophy in the studied organs, with impressive results in gingiva (capillaries, $p < 0.01$) and heart (muscle fibers, $p < 0.001$), except the liver (centrilobular vein, $p > 0.05$). Recorded hypergrowth did not differ significantly between sexes in Group B1, regardless of the organ ($p > 0.05$), but 3 times more females died within Phase 1, due to very-high dose Nifedipine toxicity. After Phase 2, hypertrophy reduction was statistically significant in Group B2 (Hydrocortisone) compared with Group B1 (Nifedipine), in gums, heart (muscle fiber diameter) and kidneys ($p < 0.05$), but still a noticeable level of inflammation persisted in gum and heart in comparison with control Group A2 ($p < 0.05$). In the kidney, HC-nano anti-inflammatory effect was potent enough to obtain no statistical differences between Groups B2 and A2 ($p > 0.05$). **Conclusions:** Very-high dose medium-term Nifedipine induces similar hypertrophic effects like chronic administration. Hydrocortisone-loaded nanoparticles have potent effects on several tissues in rats.

Keywords: nifedipine, hypertrophy, gingiva, hydrocortisone, nanoparticles.

INTRODUCTION

Inflammation is often characterized by hypertrophy and consists in the activation of immune and nonimmune cells. They protect the host organism from bacteria, viruses, toxins, and infections by eliminating these pathogens, and also can promote tissue recovery and repair [1, 2]. Recent studies reveal that the absence of inflammatory symptoms is not the end of

innate immune responses to infections. There is additional immunological activity that occurs after completion of symptoms that alter the immune physiology of tissues. This is called adapted homeostasis [3].

In the present study, our interest is focused on the dento-maxillary system, mainly gingival hyperplasia, that was experimentally induced with the help of a very high dose of Nifedipine, using an

endogastric tube as administration technique. Excessive and uncontrolled gum growth is called gingivitis or gingival hyperplasia. Gingival hyperplasia may be a side effect of administering certain classes of drugs without necessarily targeting the gum, such as calcium channel blockers, immunosuppressants or anticonvulsants [4]. Nifedipine is a first-generation calcium channel blocker that is commonly prescribed in cardiac pathologies. As a side effect, it can induce gum inflammation, especially on long term usage, causing hypergrowth of both gingival epithelium, and dental pulp [5].

Gingivitis is clinically manifested by speech and mastication disorders, due to sensitivity which can exacerbate towards extreme pain, encompassed by bleeding. The latter determines the patient to avoid daily brushing, increasing the risk of tooth decay and periodontal disease, which complicates with hypermobility and even loss of teeth [6].

Nanotechnology has opened many opportunities in dentistry, with diverse applications in several oral pathologies, that can represent a major concern for public health worldwide. Oral diseases like cavities, gingivitis, periodontitis, and even oral cancer can affect large categories of people, regardless of age. Unhealthy eating habits, personal medical history and inherited factors can aggravate oral problems [9].

The gingival hypergrowth of the study animals in our experiment was treated with liposomes loaded with Hydrocortisone (HC-nano). Recent studies advocate for liposomes (LPs) use as nanocarriers being lipid-based, biocompatible and biodegradable spherical vesicles capable of transporting drugs and targeting specific tissues [10]. In literature, LPs have variable properties due to their size (60-100 nm) and chemical structure, based on a hydrophobic portion that is placed between the two lipid layers [11, 12, 13, 14]. Lipid-loaded nanoparticles have a multitude of

advantages because of their biocompatibility and adaptability, necessary for traveling in the human body. Steingoetter et al. demonstrated that the human body's lipid processing capacity is similar like that of the rats. Therefore, the results from studies using animals *in vivo* are relevant and open future scientific research [15].

Hydrocortisone (HC) is a steroid hormone most often recommended as a powerful anti-inflammatory in patients with several inflammatory conditions, autoimmune diseases, and adrenal insufficiency [16]. Although corticosteroids are a water-insoluble drug molecule with low bioavailability, it can be greatly improved by inclusion complexes [17, 18]. Slominski et al. state that the variety of important aspects of steroidogenesis, secosteroidogenesis and their role in the context of inflammation and immunity still needs to be studied, leading to practical and clinical solutions for several inflammatory and autoimmune pathologies [19].

We incorporated HC into nanoparticles because it is the only solution to entirely protect the medicine in case of administration on an endogastric tube, and thus to have the anti-inflammatory effect without being hindered by physiological barriers of the body.

We aim to study organ penetrability and anti-inflammatory effect of Hydrocortisone-loaded nanoparticles (HC-nano) after inducing hypertrophy with previous very-high dose medium-term Nifedipine administration. The latter can replicate a long-term effect of the regular dose ingestion of the calcium channel blocker.

MATERIAL AND METHODS

Nifedipine administration

To accelerate the hypertrophic side effect of Nifedipine (Sigma Aldrich), we needed to administer it in a very-high dose

(approximately 100 times higher than therapeutic human dose, but 10 times below LD50 in rat) of 100 mg/kg body weight daily. This is equivalent to the dose of 10 mg/100g of rat. Thus 10 mg amount of Nifedipine was dissolved in 0.2 mL *a.d.* of Normal Saline - 0.9% (Braun), therefore obtaining an oral Nifedipine solution with a concentration of 5%. The prepared solution was given through an endogastric tube, twice daily (a 200g-rat receives 0.2 mL every 12 hours).

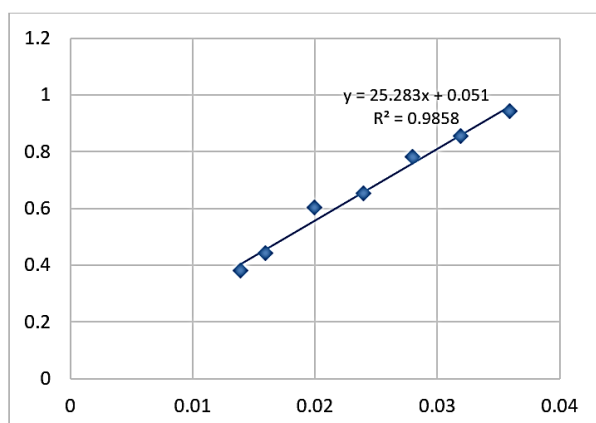
Preparation of Hydrocortisone-loaded nanoparticles (HC-nano)

In order to load Hydrocortisone into LPs we used the thin-film hydration method [20]. Initially 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti) DSPC (120mg) and Cholesterol (CHOL, Sigma Aldrich) (80mg) were dissolved in a 1:1 (v/v) mixture (4mL/4mL) of Chloroform (Sigma Aldrich) / Methanol (Sigma Aldrich). These substances did not need any additional purification, as they were of analytical grade. We extracted the solvents from a 100-mL round bottom flask by evaporation technique using a rotary (60 rpm) evaporator (Heidolph Laborota 4002) with reduced pressure at 35 degrees Celsius. We obtained a thin lipid film on the glass wall of the container, after which we hydrated it with 30mL of Hydrocortisone-

hydrogensuccinate (Zentiva) (11,35 mg/mL), at room temperature. We applied 10 minutes of intense vortex shaking which led to Hydrocortisone-loaded multilamellar vesicles (HC-nano-MLVs) formation. To strengthen the homogeneity of the liposome-suspension, it was introduced in a sonication bath for 15 minutes, followed by 2 hours of resting time. The HC-nano-MLVs were purified from non-encapsulated remanent medicine using successive cycles of washing/centrifugation for 10 minutes at 15000 rpm.

Calibration of HC-nano

As explained above, in order to obtain LPs loaded with HC, the lipid film required hydration with 30 ml of HC solution (11.35 mg/mL). To determine the amount of HC included in the LPs, we used the indirect method of estimation for the LPs suspension that was centrifuged, and the supernatant was separated and analyzed spectrophotometrically (253 nm). The determination of HC in LPs was performed by breaking the liposome vesicles with a nonionic surfactant, Octylphenol Ethoxylate (TRITON X-100 Surfactant, Dow Chemical), and the spectrophotometric determination of the HC was carried out by reporting the values obtained to the calibration curve shown in figure 1.



Conc. (mg/ml)	Abs
0.2	1.255
0.04	0.954
0.036	0.941
0.032	0.858
0.028	0.779
0.024	0.653
0.02	0.602
0.016	0.443
0.014	0.379

Fig. 1. Hydrocortisone (HC) calibration curve following breakage of HC-loaded liposomes using a nonionic surfactant.

The amount of HC included in the LPs was determined by making the difference between the initial value and the actual one, namely 9.15 mg. The encapsulation efficiency of the tested formulation was calculated considering 9.15 mg HC entrapped and 11.35 mg of HC solution used for phospholipid film hydration,

resulting in an efficiency of encapsulation of 80 %. The efficiency of encapsulation (EE) was calculated with equation no. 1. The drug/lipid (DL) ratio was calculated considering 9.15 mg HC and 120 mg of phospholipid content (PC) at every 1 mL liposomal suspension (Drug/lipid = 9.15 mg HC/120 mg PC).

$$EE \% = \frac{\text{Encapsulated HC (mg)}}{\text{Total HC (mg)}} \quad [1]$$

$$DL \% = \frac{\text{Encapsulated HC (mg)}}{\text{Total PC (mg)}} \quad [2]$$

Ethical standards and experimental animals

This experiment was conducted in two phases on 60 adult, healthy Wistar rats, respecting the formal approval from the Ethics Commission (Certificate No. 93/14.06.2021) of Grigore T. Popa University of Medicine and Pharmacy, Iasi.

The animals had a sex ratio of 1:1 (males : non-pregnant females) and were acquired from Cantacuzino National Medical Military Institute for Research and Development in Bucharest.

All animal subjects did not undergo any genetic modifications and were aged 3 months at the beginning of Phase 1 in our experiment. Their initial weight interval was 200 - 250 grams, and daily exact weighing was necessary due to the very-high-dose of Nifedipine administration. Daily nutrition was balanced in terms of energy and nutrients intake. Laboratory standard conditions (25°C temperature / 60% air humidity) were guaranteed by the Advanced Center for Research and Development in Experimental Medicine (CEMEX) of Grigore T. Popa University of Medicine and Pharmacy Iasi.

Experiment phases

This experiment was conducted in two phases on our 60 Wistar rats. In Phase 1, the animals were randomly divided initially in two groups, respecting a ratio of 1:1 between males and non-pregnant females, within each group.

Group A1 was deemed control, and included 20 rats (10 males, 10 females) that did not receive any medication. Group B1 consisted of 40 rats (20 males, 20 females), and received oral Nifedipine in a very high dose (approximately 100 times higher than therapeutic human dose, but 10 times below LD50 in rat) of 100 mg/kg body weight daily, for 4 weeks, diluted in solution (Normal Saline, 0.9%, B. Braun Medical) and using an endogastric tube.

After Phase 1 ended, 40% of each sex, within each group of rats were sacrificed. Different organs were harvested and analyzed microscopically for any modifications in structure. However, Nifedipine toxicity induced death in an unequal number of females vs. males during this phase, in Group B1. This is the

reason for which the up-mentioned percent (40%) of sacrificed rats needed to vary a little bit among each sex (42-44%), in each group, due to the mathematical approximation to nearest integer.

The resulting two groups of living rats entered in Phase 2, and names were changed to A2 and B2, respectively. Group A2 (control) had 12 rats (6 males, 6 females) and continued to receive no medication, while Group B2 counted 18 rats (10 males, 8 females) and were given liposomes loaded with Hydrocortisone (HC-nano) in human average therapeutic dose (2 mg/kg body weight daily), through the endogastric tube, for another 4 weeks. After the completion of Phase 2, all rats were sacrificed, and tissues were inspected under the optical microscope with different magnifications.

Specimen sampling and microscopical analysis

We harvested the following organs: gums, teeth, heart, liver, and kidneys. Soft tissue conservation was obtained in paraformaldehyde containers. Following fixation by glycerol-resins mixture that prevent formation of ice crystals, and freezing at -40°C , tissue sections were cut with Leica CM1860UV microtome (5.0 μm slices, at -16°C). Meyer albumin was used on the glass slides when placing the sections.

To study the Nifedipine-induced hypertrophy and HC-nano anti-inflammatory effect on teeth, it was necessary to decalcify the hard dental structure. Decalcification (based on 2% Formic acid and 20% Sodium citrate, in 1:1 vol. solution) is necessary for microscopic examination of hard tissues, being a routine procedure that lasted 10 days and finalized with washing (water) for 24 hours.

Microscopy was performed mainly in coloration with Hematoxylin-Eosin dye, but for specific histological details, we also used the following dyeing techniques:

Gömöri Silver impregnation, Weigert Ferric Hematoxylin staining, Van Gieson trichromic staining, Goldner-Szeckelly trichromic (TGS) staining, and Periodic Acid-Schiff (PAS) staining.

Tissue inspection under optical microscopy (Leica DMC2900) took place at the Clinical Emergency Hospital for Children "Saint Mary" in Iasi.

In order to observe the changes caused by Nifedipine administration (Phase 1) and subsequent administration of HC-nano (Phase 2), the size of organ fragments (length and width) was measured at different magnification power (x100, x200, and x300), depending on the studied organ fragment.

Data analysis

We processed the recorded data sets with SPSS 18.0 Software, using the optimal tests for descriptive and analytical statistics, implemented at a 95% CI. Average values were calculated together with dispersion indicators. Assessment for significant difference comparing two groups with normal distributions, was done using t-Student Test. A $p \leq 0.05$ was indicative for a 95% statistical significance.

In calculating the difference between two or more groups, at the series of values with normal distributions, at the threshold of 95% significance, we applied the F test (ANOVA) for quantitative variables, in conjunction with Tukey HSD (post-hoc) correction, to reduce the error rate when testing several hypotheses.

RESULTS

When Phase 1 ended, the 100 times higher than normal human dose of Nifedipine managed to induce tissue hypertrophy, which was confirmed by optic microscopy. However, the most outstanding results were observed at gingival level, not only microscopically, but also clinically (fig. 2.).



Fig. 2. Gingiva examination at the end of Phase 1, following very high-dose medium term Nifedipine administration in: **a)** Group A1 (control) - normal clinical aspect (size and color) of gingiva; **b)** Group B1 - gingival hypergrowth

During Phase 1, in Group B1, 8 rats (6 females and 2 males) have died due to the toxic effects of high dose Nifedipine. All deaths were registered between Day 4 and Day 9, and an autopsy was performed. However, minimal, or no gingival

hypergrowth was noticed (fig. 3a). The most probable death cause was the damage of the gastrointestinal tract triggered by the constipation effect of Nifedipine (fig. 3b). In control Group A1 all rats lived to finish Phase 1.



Fig. 3. Autopsy of the first female rat who died in Group B1 on Day 4 of Phase 1: **a)** no gingival hypergrowth; **b)** intestines were highly distended and filled with air

When Phase 2 started, Group A2 consisted of 12 rats (6F/6M), which resulted from the sacrificing of 8 rats (4F/4M) at the end of Phase 1. Group B2 was formed by the remaining 18 rats (8F/10M) of Group B1, minus the encountered deaths and the needed scarification for microscopic evaluation purpose of tissues.

When Phase 2 ended, the HC loaded nanosystems managed to reverse the tissue hypertrophy partially in some organs, and almost entirely in others, findings that were confirmed by optic microscopy. In what concerns the gingiva, at clinical exam, a small grade of hypergrowth was still noticed in most of the rats (fig. 4), before sacrifice had to occur in the end.

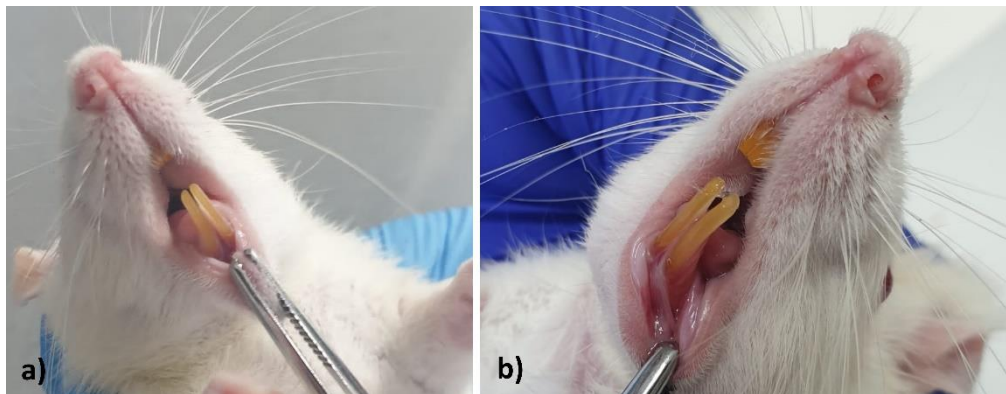


Fig. 4. Gingiva examination at the end of Phase 2, following HC loaded nanoparticles administration in: **a)** Group A2 (control) - normal clinical aspect of gingiva remained unchanged; **b)** Group B2 - gingival hypergrowth has reduced, but still one can notice size and color differences with Group A2

After inducing hypertrophy with Nifedipine, the following histological aspects have emerged in several organs. At the gum level, the degeneration of muscle

fibers is observed, and they have an increased volume, while the epithelial cells become more and more superficial and are loaded with keratin (figs. 5a, 6a, 6b).

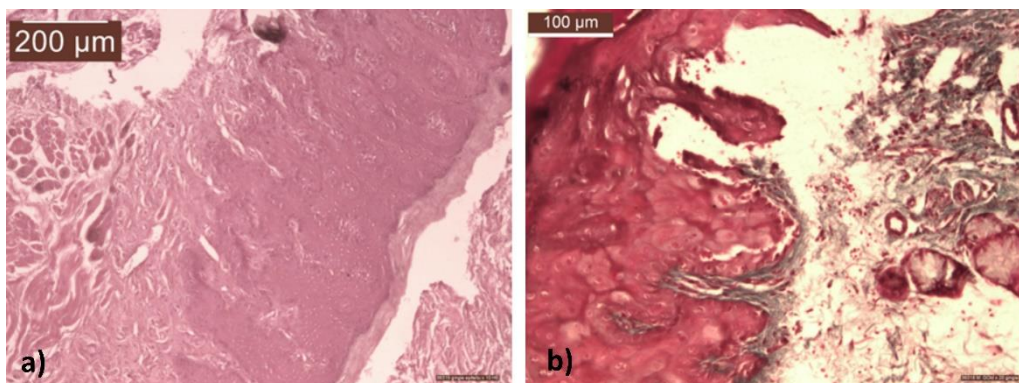


Fig. 5. Microscopic image of gum tissue, comparison between end of Phase 1 vs. end of Phase 2: **a)** gingival muscular and epithelial hypertrophy, the latter is showing keratin deposits, following Nifedipine administration; immunohistochemical staining HE (x100); **b)** partial hypertrophy reversal after HC-nano, using Goldner-Szeckelly trichromic (TGS) staining; keratinized gingival epithelium is appearing red and muscle fibers are seen in green-grey (x200)

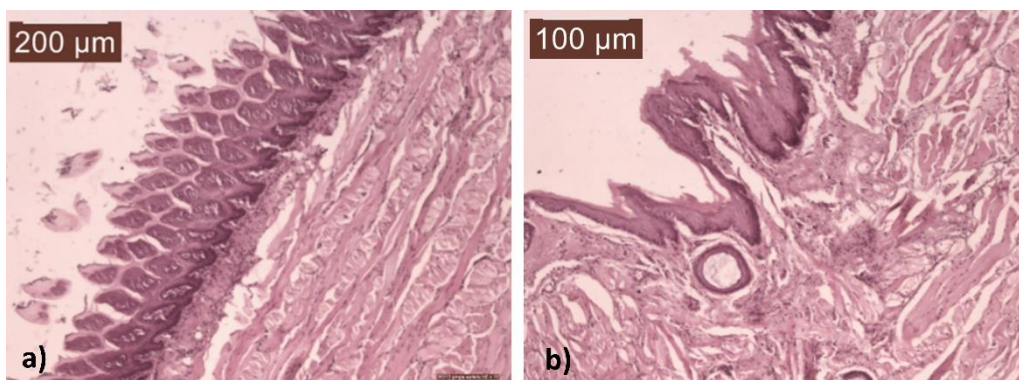


Fig. 6. Microscopic image of gum tissue at the end of Phase 1, using immunohistochemical staining HE: **a)** prominent keratin deposits (x100); **b)** hyperplasia of gingival epithelium (x200)

HC-nano acted at the gingival level, determining a satisfactory, but not complete reduction in the hypergrowth (figs. 5b, 7a, 7b, 8a, 8b). Figure 5 (a vs. b) presents a side-by-side comparison between, end of Phase 1 vs. end of Phase 2, at this level.

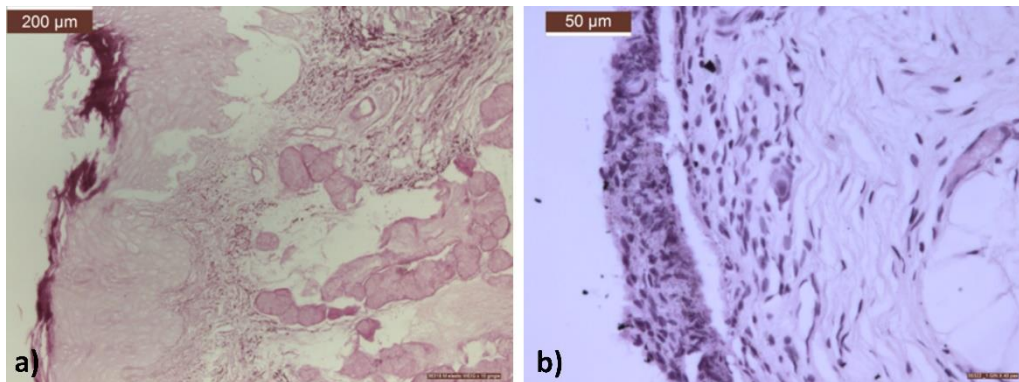


Fig. 7. Microscopic image of gum tissue at the end of Phase 2, following HC-nano administration: **a)** using Weigert Ferric Hematoxylin Staining; presence of keratinized gingival epithelium is still noted (x100); **b)** using Periodic Acid-Schiff (PAS) staining; a decrease in the size of the gingival epithelium and reduced presence of inflammatory cells (x400)

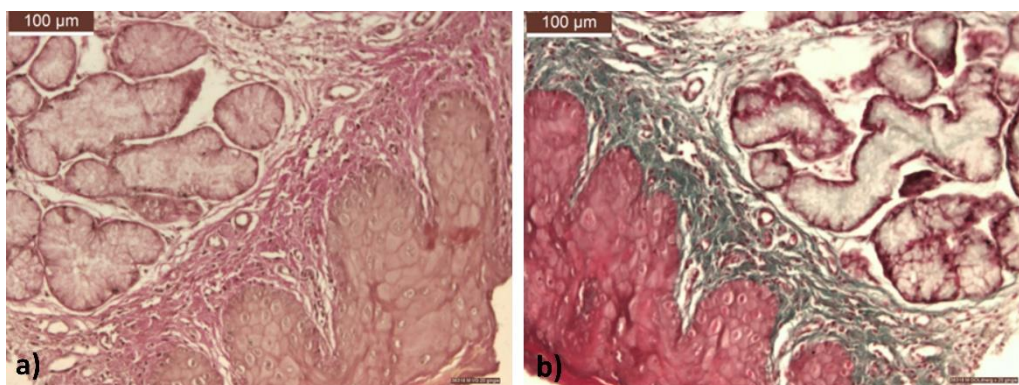


Fig. 8. Microscopic image of gum tissue at the end of Phase 2, following HC-nano administration: **a)** using Van Gieson trichromatic staining; one can note the keratinized gingival epithelium (brown, inferior-right side of image), hypertrophied muscle fibers (red, middle diagonal of image) and affected salivary glands (pale pink, superior-left side of image) (x200); **b)** using Goldner-Szeckelly trichromatic staining (TGS) after HC-nano administration; the keratinized gingival epithelium (red) is noted, the muscle fibers are colored hypertrophied (green) and the affected basement membrane (dark red) (x200)

Following the microscopy preparation with decalcification, we outlined the hypertrophied dental pulp at the end of Phase 1 (fig. 9a), and the damage of both dental pulp and the rest of hard dental tissue, when Phase 2 ended (fig. 9b).

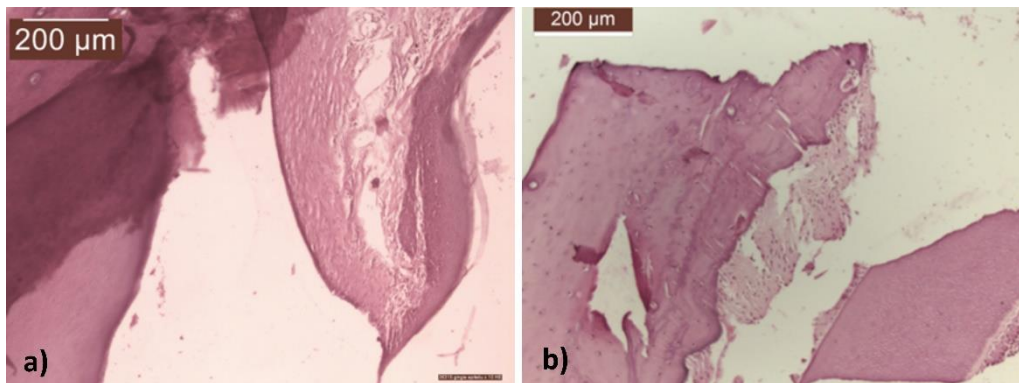


Fig. 9. Microscopic image of the tooth using immunohistochemical staining HE: **a)** after administration Nifedipine, there is a loss of normal architecture, with abnormalities in the dental pulp (x100); **b)** after HC-nano administration, it is noted the destruction of the dental pulp, and of the dental tissue (x100)

Nifedipine affected heart tissue and muscle hypertrophy is also present at this level (fig. 10a). Regarding the nerve fibers that innervate the heart, they appear with altered dimensions, due to a demyelinated aspect. After the completion of Phase 2, the effect of the anti-inflammatory resulted in a decrease in the diameter of muscle fibers (fig. 10b).

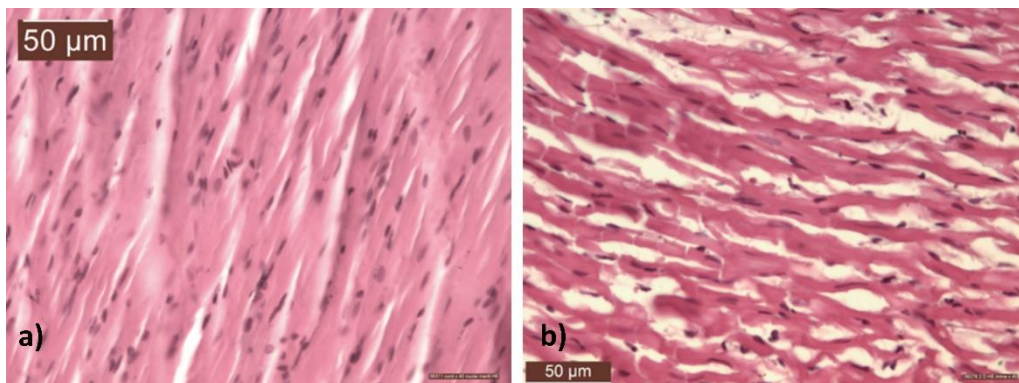


Fig. 10. Microscopic image of heart tissue: **a)** using immunohistochemical staining HE after administration of Nifedipine. It is noted the presence of enlarged nuclei, loss of myocardial striae (x400); **b)** using immunohistochemical staining HE after HC-nano administration. It is noted the decrease in the size of muscle fibers (x400)

The hepatic lobule stands out (fig. 11a) with the centrilobular vein. In the periphery, the bile ducts begin to have their own wall, and the spaces between the sinusoid capillaries became larger, meaning increased blood flow to the liver, which can be correlated with Nifedipine administration. Following administration of HC-nano, the centrilobular vein decreased slightly in diameter, but some hepatocytes appear destroyed, and liver nuclei are no longer so obvious, or they even disappear (fig. 11b).

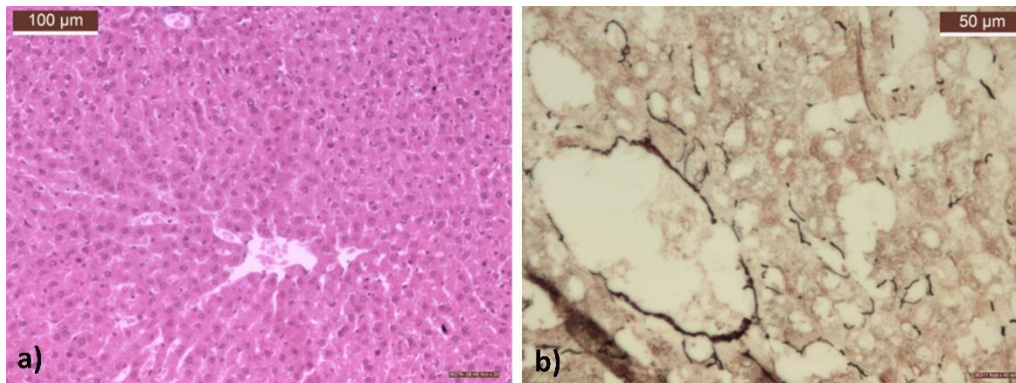


Fig. 11. Microscopic image of liver tissue: **a)** using immunohistochemical staining HE after administration of Nifedipine; centrilobular vein is seen approximately in the center of the image, spaces between the sinusoid capillaries became larger (x200); **b)** using Gömöri silver impregnation after HC-nano administration, reticulin deposits (intense black) can be identified, as well as the basal membrane (x400)

Regarding kidney tissue, we observed damage at the renal glomeruli, most probable due to the very high dose of Nifedipine (fig. 12a). These modifications were persistent also at the end of Phase 2; the renal glomeruli reflect areas of necrosis, with the decrease in the number of nuclei at their level, up to their disappearance (fig. 12b).

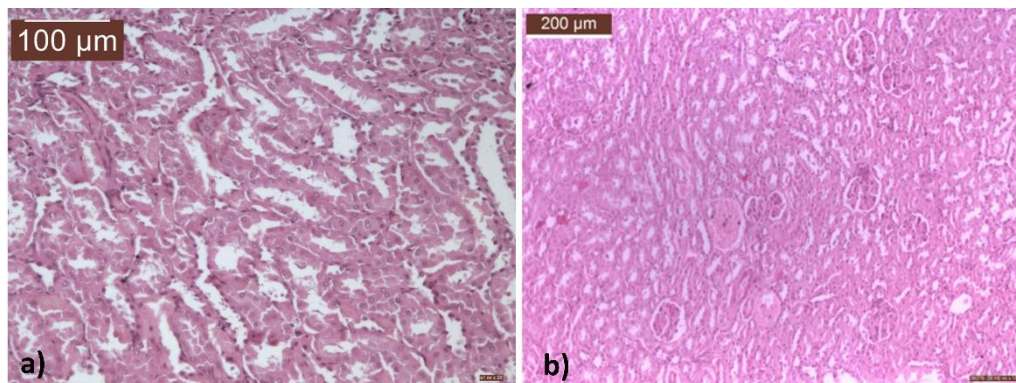


Fig. 12. Microscopic image of kidney tissue using immunohistochemical staining HE: **a)** necrosis of the uriniferous tubules and the appearance of deposits in the lumen at the end of Phase 1 (x200); **b)** after Phase 2 necrosis of renal glomeruli remained positive (x100)

After Nifedipine administration in Phase 1, statistically significant hypertrophy was observed in the studied organs, with outstanding results at gingival level in the dimensions of capillaries ($p=0.009$ for D1; $p=0.007$ for D2) and heart muscle fibers ($p<0.001$), while in liver, the recorded increase was not significant ($p>0.05$) (tab. I).

TABLE I.
Measured hypergrowth at the end of Phase 1 in studied organs,
in Group A1 (control) vs Group B1 (Nifedipine)

Organ	Length/D1 (μm)			Width/D2 (μm)		
	Group A1	Group B1	P*	Group A1	Group B1	P*
Gum - magnification power x200 ("100 microns")						

Muscle fibers x±SD, limit	18.55±0.88 17.53 - 19.86	48.89±4.94 20.00 - 73.33	0.017	8.24±0.59 7.26 - 9.11	15.00± 1.33 13.33 - 16.87	0.021
Capillaries x±SD, limit	3.48±0.71 2.61 - 4.55	31.02±5.29 5.00 - 153.33	0.009	1.96±0.45 1.47 - 2.66	20.20±3.39 3.33 - 80.00	0.007
Kidney - magnification power x200 ("100 microns")						
Renal glomerulus x±SD, limit	76.11±0.70 75.22 - 77.13	88.66±18.20 64.66 - 113.33	0.041	65.78±0.75 62.77 - 66.79	83.33±20.41 57.32 - 105.66	0.039
Liver - magnification power x200 ("100 microns")						
Centrilobular vein x±SD, limit	90.66±24.09 63.33 - 136.55	107.67±32.64 70.22 - 145.55	0.099	63.66±14.61 46.47 - 85.23	71.20±21.08 48.77 - 95.55	0.319
Heart - magnification power x400 ("50 microns")						
Muscle fibers x±SD, limit	20.53±0.66 19.38 - 21.55	43.78±16.65 23.33 - 63.12	0.001	8.27±1.01 7.02 - 9.55	18.18±6.60 8.33 - 28.33	0.001
* F _{ANOVA} test D1 (Diameter 1) ≥ D2 (Diameter 2)						

In the results from the comparison by sex, at the end of Phase 1, in both groups, we did not observe any differences in the control group, whereas in Group B1, there are some small differences in the measured dimensions of analyzed tissular structures, but with no statistical significance (tab. II).

TABLE II.
Comparison of tissue hypertrophy by sex in both studied groups, at the end of Phase 1

Organ	Group A1 - control			Group B1 - after Nifedipine		
	Males x±SD (µm)	Females x±SD (µm)	t-Student test	Males x±SD (µm)	Females x±SD (µm)	t-Student test
Gum - Muscle fibers , magnification power x200 ("100 microns")						
Length/D1	18.45±0.90	18.65±0.95	0.742	50.94±8.15	38.33±11.41	0.507
Width/D2	8.17±1.40	8.82±1.39	0.695	16.57±7.17	14.47±9.38	0.764
Kidney - Renal glomerulus , magnification power x200 ("100 microns")						
Length/D1	76.18±0.77	76.05±0.70	0.790	98.25±24.75	82.0±21.81	0.653
Width/D2	65.86±0.81	65.71±0.78	0.764	87.83±15.66	80.13±18.26	0.289
Liver - Centrilobular vein , magnification power x200 ("100 microns")						
Length/D1	92.83±23.11	90.25±21.53	0.848	109.53±33.72	101.41±31.18	0.589
Width/D2	65.68±14.23	62.08±13.29	0.795	75.86±22.17	68.95 ±20.39	0.452
Heart - Muscle fibers , magnification power x400 ("50 microns")						
Length/D1	20.46±0.70	20.59±0.68	0.771	49.58±15.76	41.87±16.21	0.689
Width/D2	8.33±1.07	8.20±1.23	0.857	18.88±8.55	17.91±6.41	0.841
D1 (Diameter 1) ≥ D2 (Diameter 2)						

The gum hypertrophy after high dose Nifedipine was objectified by the increased average dimensions of both capillaries (greater diameter, D1, from 3.48±0.71µm to 31.02±5.29µm; p=0.009), and, also of the muscle fibers length (18.55±0.88µm vs 48.89±4.94µm; p=0.017) and width (8.24±0.59µm vs 15.00±1.33µm; p=0.021). After treatment with HC-nano, the greater diameter of gum capillaries reduced their

size with statistical significance from 31.02±5.29µm to 24.79±6.13µm. However, the size remained very raised in comparison with control Group A2 (3.52±0.69µm) (tab. III).

Mean level of length of heart muscle fibers increased in Phase 1, from 20.53±0.66µm to 43.78±16.65µm, and in Phase 2, it decreased to 35.71±16.98µm, presenting the strongest statistical

differences in Phase 1 and no significance, when we compare the level of reversal in Phase 2 ($43.78 \pm 16.65 \mu\text{m}$ vs $35.71 \pm 16.98 \mu\text{m}$) (tab. III).

Regarding the liver tissue, we measured the diameters of the centrilobular

vein, and even if greater diameter slightly increased following Nifedipine administration ($90.66 \pm 24.09 \mu\text{m}$ to $107.67 \pm 32.64 \mu\text{m}$), and then it decreased to $91.65 \pm 27.43 \mu\text{m}$, all these variations did not present any statistical significance (tab. III).

TABLE III.
Hypergrowth reversal at the end of Phase 2 in studied organs,
in Group A2 (control) vs Group B1 (Nifedipine) vs Group B2 (HC-nano)

Organ	Length/D1 (μm)			Width/D2 (μm)		
	Group A2	Group B1 B1 vs A2	Group B2 B2 vs A2, B2 vs B1	Group A2	Group B1 B1 vs A2	Group B2 B2 vs A2, B2 vs B1
Gum - magnification power x200 ("100 microns")						
Muscle fibers	18.64 ± 0.92	48.89 ± 4.94 ^{c)}	25.00 ± 8.10 ^{c), c)}	8.29 ± 0.73	15.00 ± 1.33 ^{c)}	13.33 ± 4.21 ^{c), d)}
x \pm SD, limit	17.49 - 19.84	20.00 - 73.33	16.3 - 46.66	7.37 - 9.23	13.33 - 16.87	8.97 - 23.28
Capillaries	3.52 ± 0.69	31.02 ± 5.29 ^{b)}	24.79 ± 6.13 ^{b), c)}	2.02 ± 0.63	20.20 ± 3.39 ^{b)}	14.34 ± 4.45 ^{b), c)}
x \pm SD, limit	2.68 - 4.75	5.00 - 153.33	4.9 - 98.0	1.35 - 2.78	3.33 - 80.00	6.25 - 59.17
Kidney - magnification power x200 ("100 microns")						
Renal glomerulus	75.34 ± 0.80	88.66 ± 18.20 ^{c)}	78.62 ± 9.04 ^{d), c)}	63.43 ± 0.82	83.33 ± 20.41 ^{c)}	70.29 ± 12.43 ^{d), c)}
x \pm SD, limit	73.82 - 76.98	64.66 - 113.33	65.22 - 97.13	62.13 - 65.23	57.32 - 105.66	55.86 - 87.35
Liver - magnification power x200 ("100 microns")						
Centrilobular vein	88.87 ± 25.11	107.67 ± 32.64 ^{d)}	91.65 ± 27.43 ^{d), d)}	59.84 ± 15.38	71.20 ± 21.08 ^{d)}	65.89 ± 16.69 ^{d), d)}
x \pm SD, limit	59.73 - 125.83	70.22 - 145.55	60.22 - 125.14	42.75 - 92.26	48.77 - 95.55	47.05 - 88.72
Heart - magnification power x400 ("50 microns")						
Muscle fibers	21.35 ± 0.72	43.78 ± 16.65 ^{a)}	35.71 ± 16.98 ^{c), d)}	8.87 ± 1.13	18.18 ± 6.60 ^{a)}	12.59 ± 4.5 ^{b), c)}
x \pm SD, limit	19.65 - 23.10	23.33 - 63.12	17.54 - 57.36	7.33 - 10.12	8.33 - 28.33	7.98 - 18.23
F _{ANOVA} test Tukey HSD post-hoc test: a) p<0,001; b) p<0,01; c) p<0,05; d) p>0,05						
D1 (Diameter 1) \geq D2 (Diameter 2)						

DISCUSSION

Inflammation treatment targets a broad set of mediators that regulate cellular manifestations necessary to remove inflammatory cells and downgrade the systemic response to restore normal tissue function [21]. Inflammation is characterized by hypertrophy, neuroendocrine and metabolic changes, depending on both the evolution of the inflammatory response, and tissue location [22, 23]

Studies conducted by Straub RH et al. determined that a normal inflammatory response is characterized by a small/moderate increase in inflammatory activity over time, in relation with an inducing danger of any kind (mechanical, thermal, chemical, biological), and then disappears

when the danger has been removed [24, 25]. A link has been demonstrated between the presence of certain psychological, biological, environmental factors and the disappearance of inflammation, as well as the development of a state of low-grade, non-infectious chronic systemic inflammation [26, 27].

Nifedipine is an antihypertensive drug widely used in chronic cardio-vascular pathologies, in both regular and long-acting forms [28], and also in pregnancy-related hypertension, which is the commonest disorder encountered in approximately 10% pregnant women [29]. It is a first generation dihydropyridine L-type calcium channel blocker, first developed by Bayer, and described in medical literature in 1972 [30].

Dongari-Baqtzoglou published data on the incidence of gingival hypertrophy induced by chronic Nifedipine administration that can be as high as 20% in humans [6], compared to a third-generation calcium channel blocker such as Amlodipine, which shows a reduced incidence ranging from 1.7% to 3.3% [7,8]. Regarding the hypertrophic side-effects in pregnancy, the literature lacks data, probably because it is given to patients for a limited period, especially in the third semester. Brochet et al. reported gingival hyperplasia associated with Nifedipine in the case of a 27-year-old pregnant patient [31].

We designed our experiment into two phases, and we aimed to reproduce the hypertrophic side effects of Nifedipine, observed in patients who take this medicine chronically. The literature emphasizes the need to distinguish drug induced inflammation, as pharmacological side effect, and other possible inflammatory conditions [23, 32]. Regarding the latter, inflammation is specific to a wide array of pathologies ranging from pediatric rheumatic diseases [33], to inflammatory pathologies specific to adult age, like rheumatoid arthritis, periodontal disease [34] or oncologic conditions [35]. In what concerns our experiment design, we decided to implement control groups and to induce chronic hypertrophy with Nifedipine, in a limited amount of time of 4 weeks, by administering a very high dose of approximately 100 times higher than therapeutic human dose [30]. This chosen dose of 100 mg/kg body weight daily was also thought to be 10 times below LD50 in rat (1022 mg/kg body weight) [36], to allow optimum survival throughout the experiment.

However, 8 rats died due to the toxicity related to the high dose of Nifedipine. Mortality among females was higher compared to male rats (6 females and 2 males), and deaths happened between the 4th and the 9th day of Phase 1. We

performed autopsy in all rats who died, and we observed the dilated abdominal cavity, very hard at palpation, with presence of highly distended intestines (fig. 3b). Clinically, these subjects suffered constipation immediately after Nifedipine treatment initiation, and in the following days they stopped defecating. The animals lost appetite and stopped ingesting food and liquids, and before death occurred, they showed acute signs of dehydration. We can conclude that occlusion of the gastrointestinal tract was triggered by Nifedipine constipation effect [28]. The death rate of 3:1 (F:M), determined us to perform microscopic comparison based on sexes for tissues in the sacrificed rats at the end of Nifedipine trial. We found no differences in control group A1, while in the experimental Group B1, we identified only small differences, but with no statistical significance (tab. II). Regarding the expected gingival growth, it was in fact minimal, or no hypertrophy was seen in these 8 deceased rats (fig. 3a), and we assume that less than 10 days of high dose Nifedipine is unable to trigger inflammation at this level. An interesting future research would be to correlate the effect of Nifedipine with the activity of interstitial cells of Cajal (ICCs) that can be found within the smooth muscle cells of the gastro-intestinal tract and can be involved in slow intestinal movements [37].

After inducing hypertrophy at the gum level, we can correlate the degeneration of enlarged muscle fibers and the aspect of gingival mucosa loaded with keratin with the objective microscopic measurements of both muscle fibers and vascular capillaries. The average dimensions of the muscle fibers grew significantly with 163.5% in length (from $18.55 \pm 0.88 \mu\text{m}$ to $48.89 \pm 4.94 \mu\text{m}$, $p=0.017$), and in width with 82.0% (from $8.24 \pm 0.59 \mu\text{m}$ to $15.00 \pm 1.33 \mu\text{m}$, $p=0.021$). The diameters of capillaries are the ones that enlarged the most, in terms of growing percentage (more than 7 times, $p<0,01$)

(tab. I), as it has been reported [4, 6] and is being consistent with classical theories of inflammation [32].

Periodontal disease is defined by affecting at least one of the supporting elements of the tooth. Gingival hyperplasia, if not treated, can easily develop into periodontitis, that is irreversible, and evolves over time towards alveolar bone damage and tooth loss [38]. This complication has deep implications in both patient appearance, from an anthropomorphic point of view, and also from the healthcare costs to rehabilitate the dento-maxillary functions [39]. During microscopic visualization of dental tissue, we observed the modifications of hypertrophied dental pulp at the end of Phase 1 (fig. 9a).

In our experiment, the other tissue that showed hypertrophy with the highest statistical significance ($p=0.001$) was the heart muscle fibers. However, in terms of measuring average growth percentage (113.4% for length, 119.8% for width), the results were not as high as in the case of gum muscle fibers (tab. I). We can explain this significant growth relating it with the high reactivity of myocardium to infectious or non-infectious agents, ranging from drugs, streptococcal infections, viral infections, and even post-viral status with immune mediated response, like in the case of COVID-19 infections [40, 41].

In Phase 1, the induced enlargement to renal glomerulus was modest but statistically significant, with average diameters lengthening with 16.4% (greater diameter, $p=0.041$) and 26.6% (lesser diameter, $p=0.039$) (tab. I). When measuring a structure microscopically, if it is not a perfect sphere, the several sections of the structure will display as an oval with two diameters, depending on the relative angle of the microtome cutting. In liver, the tissue did not display histologic modifications (fig. 11a) and the dilatation of the centrilobular vein (18,7% at D1; 11,8% at D2) was not significant ($p>0.05$)

(tab. I). Our findings are in line with the safety renal and hepatic profile of Nifedipine from literature; very few case reports of acute interstitial nephritis, or hepatitis being reported in connection with this drug. [5, 29, 42, 43].

Nanomedicine is an area of major interest, being able to incorporate, protect and transport a certain medicine towards the targeted organ, followed by a controlled release at the destination [44]. Like introduction of lasers in medicine [33] and surgery [45], nanoparticles represent another step forward advancing the treatment possibilities of the healthcare system. However, it must be mentioned that patient health insurance usually covers classical medical and surgical treatments. The high-end therapeutic modalities, part of dental interventions, and medicines that bring a higher grade of patient satisfaction remain with costs uncovered, or partly reimbursed [46, 47]. Liposomes are colloidal systems that, due to their size of less than 1000nm, can cross capillaries easily. They can be loaded with any drug, aiming to treat multiple pathologies [48, 49].

In Phase 2 we loaded HC into nanoparticles, and we obtained a very high level of protection of the drug from the gastrointestinal barriers, when given to rats on the endogastric tube. At the end of Phase 2, HC-nano partially reversed tissue hypertrophy in gum and heart muscles, and almost entirely in kidneys and liver. These findings are supported by both the overall aspect of the examined microscopic fields, and by the measurements of the targeted structures for comparison.

In gums, the keratin deposits diminished, accompanied by reduction of the capillaries size (20.1% for D1; 29.2% for D2), and muscle fibers length (48.8%) in a statistically significant manner ($p<0.05$). Regarding the diminution of muscle fiber width, it accounted for only 11.1%, with no statistical trust ($p>0.05$) (tab. III). This behavior reveals that

previously induced Nifedipine hypertrophy remained raised and with statistical significance ($p < 0.05$) in comparison with control Group A2 (tab. III).

The anti-inflammatory action was quantified by the mean length of heart muscle fibers which decreased with only 18.4% ($43.78 \pm 16.65 \mu\text{m}$ to $35.71 \pm 16.98 \mu\text{m}$) in a non-significant manner ($p > 0.05$), being surmounted by the width reduction at 30.7% ($18.18 \pm 6.60 \mu\text{m}$ to $12.59 \pm 4.5 \mu\text{m}$), with a statistical $p < 0.05$ (tab. III).

Regarding the renal glomerulus, the initially modest, but statistically significant enlargement at a trust level of 95%, in Phase 1, was followed by a return to the average size encountered in control Group A2 (tab. III). On the other hand, the dimensions of the centrilobular vein, which were not significantly increased by Nifedipine, also underwent a reduction under the anti-inflammatory effect of HC-nano (D1 raised from $90.66 \pm 24.09 \mu\text{m}$ to $107.67 \pm 32.64 \mu\text{m}$, and reversed to $91.65 \pm 27.43 \mu\text{m}$), at a $p > 0.05$ (tabs. I and III). One can also notice that there is a very small variation in the measurements in all tissues, between the control groups A2 and A1. We expected this, since Group A2 is in fact a continuation of Group A1 into Phase 2, with a smaller number of subjects, following the sacrifice of 40% of rats at the end of Phase 1.

ETHICS AND LABORATORY ANIMALS

The Ethics Commission of Grigore T. Popa University of Medicine and Pharmacy, Iasi approved our research on animal subjects with Certificate No. 93/14.06.2021.

CONFLICT OF INTERESTS AND FUNDING

The authors declare no conflict of interest.

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Our experiment fulfilled its aims by inducing hypertrophy with very-high of oral Nifedipine for a period of 4 weeks, that was partially reversed at gingival and myocardial level, and entirely reduced by HC-nano at renal and hepatic tissues. This emphasizes the persistent hypertrophic effect our studied calcium channel blocker in some organs.

CONCLUSIONS

The importance of still studying one of the most common antihypertensives available worldwide resides in our experimental induction of chronic side effects by administering the drug in a very high dose for a “subacute” interval of time. Very high dose Nifedipine given in medium-term manages to induce similar hypertrophic effects, like the ones evocated in literature in the case of chronic administration.

Another important aspect of our experiment was to try to reverse the Nifedipine induced hypertrophy in rat tissues, with modern therapeutic approaches, like nanocarriers loaded with a steroidal anti-inflammatory drug, thus opening further research directions for extending our results to human subjects. Hydrocortisone-loaded nanoparticles have potent effects on several tissues in rat, even if gingival hyperplasia seems to be partially resistant to anti-inflammatories.

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