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Original article

The penetration of topically applied ointment containing hyaluronic acid in rabbit tissues

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Abstract

The properties of hyaluronic acid used for treatment of acute and chronic joint disease are known for many years and this compound is widely used both in humans and animals.

To obtain a therapeutic effect of a certain drug, the appropriate concentration in the target organ or tissue is important. The application of labeled compounds is one of the frequently applied techniques to estimate drug penetration into the skin and other body tissues or organs. The aim of the study was to evaluate the penetration of hyaluronic acid labeled with I-131 through the skin and its distribution within the knee joint and other internal organs in rabbits after a topical application of an ointment containing hyaluronic acid.

The experiment was performed on 22 albino rabbits divided into control and examined groups. Fifteen rabbits were exposed to the multicomponent ointment containing hyaluronic acid labeled with I-131. Time of exposure was 48 hours.

Hyaluronate penetrated to a high degree into the examined tissues. No significant differences in terms of leg tissue activity were observed between a leg tissue exposed to labeled ointment and that unexposed, suggesting that after topical administration, the active component of the ointment is delivered to the joint via the blood stream.

Hyaluronate applied topically penetrates through the skin into the rabbit tissues and organs and into the joint fluid of both legs (exposed and not exposed). This route of administration seems to be useful for this drug delivery and allows to avoid unnecessary side effects.

Key words: hyaluronic acid, tissue penetration, rabbit, topical application

Introduction

Hyaluronan (HA), nonsulfated nonpimerized linear glycosaminoglycan (GAG) existing in vivo as polyanion of HA composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, is a widely distributed tissue extracellular matrix component and shows a variety of structural and regulatory functions which was investigated by Julovi (2011) and Santangelo (2007). Molecules of HA are generally of very high molecular mass, ranging from approximately 10^5 to 10^7 Da, but can also exist as smaller fragments. Its large size and its capacity to interact with water molecules lead to form solutions with high viscosity and elasticity that provide space filling and lubricating functions. It is a major constituent of the extracellular matrix of the skin, joints, and many other tissues and organs shown by Robert (2010). HA is involved in many biological processes, such as inflammation, embryogenesis, and wound healing according to Tammi (2002).

To obtain a therapeutic effect of a certain drug the appropriate concentration in the target organ or tissue is important (Bielecka-Grzela et al. 2003). In contrary to topical application the distribution of active agent within the body seems to be easier to predict after systemic drug administration, that is, orally, per rectum, or intravenously.

The evaluation of drug distribution after its topical application on the skin is more complicated. Several experimental methods have been elaborated for this purpose, for instance, skin blister fluid and microdialysis, particularly in the skin which is described in papers of Holmgaard (2010), Klimowicz (2007) and Olczyk (2008). Apart from these two methods, using labeled compounds is one of the techniques frequently applied to estimate drug penetration into the skin and other body tissue or organs (Bielecka-Grzela et al. 2002). In many cases investigations have to be performed in animals and quite often rabbits are chosen as experimental animals.

Phlogahyl is a multicomponent ointment containing hyaluronic acid (HA). The properties of HA for treatment of acute and chronic joint disease are known for many years, and this compound is widely used both in humans and animals.

The aim of this work was to evaluate the penetration of HA labeled with I-131 through the skin and its distribution within the knee joint and other internal organs after its topical application in rabbits.

Materials and Methods

The experiment was performed on 22 albino rabbits. They were divided into the following groups: group I, consisted of 3 rabbits; group II, consisted of

15 rabbits exposed to HA labeled with I-131 – the main ingredient of multicomponent ointment Phlogahyl; group III, composed of 4 rabbits exposed to nonlabeled ointment (the control group for background measurements). Rabbits were treated according to rules concerning experimental animals. The study protocol was approved by the ethical committee of Pomeranian Medical University. Phlogahyl's main ingredient, hyaluronic acid, was labeled with ^{131}I at the Radioisotope Centre of the Institute of Atomic Energy POLATOM in Świerk (Poland). Briefly, the labeling consisted of three steps: activation of hyaluronate, ^{131}I labeling of histamine and conjugation of ^{131}I -histamine to activated hyaluronate, effectiveness of each step was controlled by HPLC. 2 mg of sodium salt of hyaluronic acid was dissolved in 200 μl of 0.1 M MES (2-(N-morpholino)ethane-sulfonic acid), pH 6.0, and mixed with 1 mg of NHS (N-hydroxysuccinimide) and 6 mg of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and the mixture was incubated for 15 min at room temperature. To 3.65 μg of histamine hydrochloride the ^{131}I with radioactivity of 111 MBq in 20 μl of 0.1 M phosphate buffer (pH 7.4) was added, followed by 50 μg of chloramine T in 10 μl 0.1 M phosphate buffer (pH 7.4). The mixture was vortexed for 90 s and the labeling was interrupted by addition of 300 μg of sodium metabisulphite in 10 μl phosphate buffer (pH 7.4). This mixture was added to the activated hyaluronate and incubated for 2 h at room temperature. Final product was purified by HPLC chromatography on BioSep-SEC-S 4000 column using isocratic elution with phosphate buffer (Fig. 1). Fractions containing ^{131}I -hyaluronate were collected and pooled. It was precipitated by addition of 99% ethanol and centrifuged at 2500 rpm (1500 x g for 15 min). The compound was stable with labeling yield of above 95% (Fig. 2). The precipitate was then mixed with the components of Phlogahyl ointment to the final radioactive concentration of around 1.85 MBq/g.

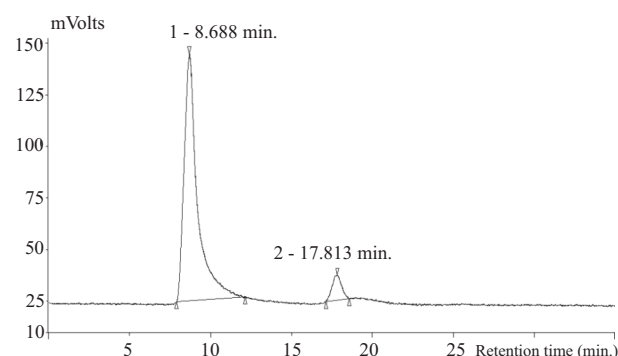


Fig. 1. Example of HPLC radiochromatogram of the reaction mixture of ^{131}I -histamine with activated hyaluronate after 2 h incubation. (1) ^{131}I -histamine-hyaluronate-93,3%, (2) ^{131}I -histamine-6,7%.

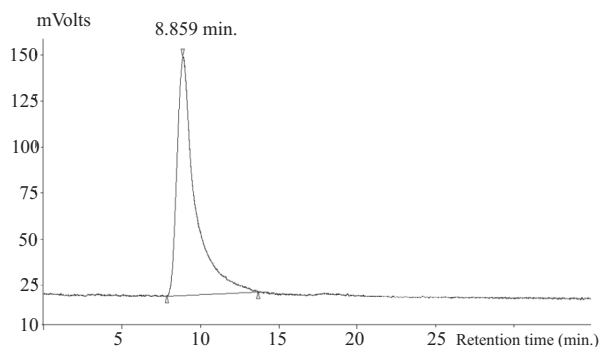


Fig. 2. HPLC radiochromatogram of ^{131}I -hyaluronate obtained from precipitate of ^{131}I -hyaluronate after reconstitution in phosphate buffer.

Separate portions of the product for every rabbit were delivered to the Department of Nuclear Medicine in Szczecin (Poland), and the activity of each portion of Phlogahyl I-131 was measured before application. Specific ointment activity varied between 1.53 and 3.66 MBq/g. Total ointment activity and weight of the sample applied on the exposed extremity were 2.24-4.63 MBq and 1.17-1.29 g, respectively. Phlogahyl I-131 was applied in 15 rabbits (group II), and time of exposure was 48 hours.

To avoid tearing off the dressing and leakage from the exposed leg and to get the comparable dose application in all rabbits during the experiment, the following procedure was performed based on a preliminary study done on 3 rabbits (group I). During the ointment application all exposed rabbits were sedated with 2 ml of Relanival (Medana Farmaterpolgrup S.A., Poland), containing 20 mg of diazepam. It was given orally approximately 15 minutes before starting the procedure. Just before the experiment, the rabbits' fur was shaved over the exposed area (knee joint and leg) down to 1 mm high for a better adherence of the ointment in the application area. Phlogahyl labeled with I-131 was applied topically on the rabbits' skin on the knee joint and leg and protected from leakage with tight dressing.

After 48 hours exposure time the rabbits were euthanized by injection of 2 ml of Morbital (Biowet Puławy, Poland) containing 26.7 mg/ml of pentobarbitalum and 133.3 mg/ml of pentobarbitalum natrium into the ear vein.

Biological samples for activity measurements were collected; each sample was placed in a separate vial. The samples of joint fluid, subcutaneous tissue, muscles, ligaments, femoral articular cartilage, tibial articular cartilage, femur bone, and skin were taken from the exposed and symmetrical (unexposed) extremities from all rabbits exposed to Phlogahyl labeled with I-131. Moreover, the following internal organ samples also were collected: liver, kidneys,

heart, lungs, thyroid gland, and blood. Specific activity expressed in counts per minute was measured for every sample in the well counter (expressed in counts per minute, but the term "activity" will be used). As previously mentioned, the activity and amount of applied ointment varied between rabbits; the specific activities of all the samples measured were normalized to 1 MBq. Additionally, to evaluate the potential harmfulness of Phlogahyl active components, the heart, lungs, kidneys, and liver samples were investigated histopathologically. Organ samples were taken and were preserved in 5% buffered formaldehyde solution. The tissues were subjected to dehydration process in increasing concentrations of alcohol, impregnated with paraffin in a tissue processor, and embedded in paraffin blocks. Sections of 3 microns thick were cut off from the paraffin blocks. The specimens prepared in this manner were stained with hematoxylin and eosin (H-E) and examined using a microscope Eclipse 600 ($\times 200$ magnification).

Statistical analysis

The arithmetical mean and the standard error of the mean were calculated and used for comparison. Extremities index (EI), defined as a ratio of activity of tissue in the exposed extremity to that of comparable tissue in the symmetrical extremity of the rabbit, and organ index (OI), defined as a ratio of organ-specific activity to that of blood, also were calculated. Because of the different amount of applied ointment and the various activities of each sample, the applied values of all parameters were normalized to 1 MBq, taking into account the activity of the individual samples of Phlogahyl labeled with I-131 and the amount of the product applied to the rabbit skin. In few cases of collected groups of parameters, the distribution differed significantly from the normal one (Kolmogorov-Smirnoff and Lilliefors tests); therefore, for a significant evaluation of the differences between the respective parameters, the Wilcoxon signed rank test was applied (Moczko et al. 2010). Level of 0.05 was assumed to be statistically significant. All the calculations were done with ProStat version 5.5 (PolySoftware International, Pearl River, USA).

Results

The mean specific activity of exposed extremities normalized to 1 MBq of the applied ointment, expressed in counts per minute, is presented in Fig. 3.

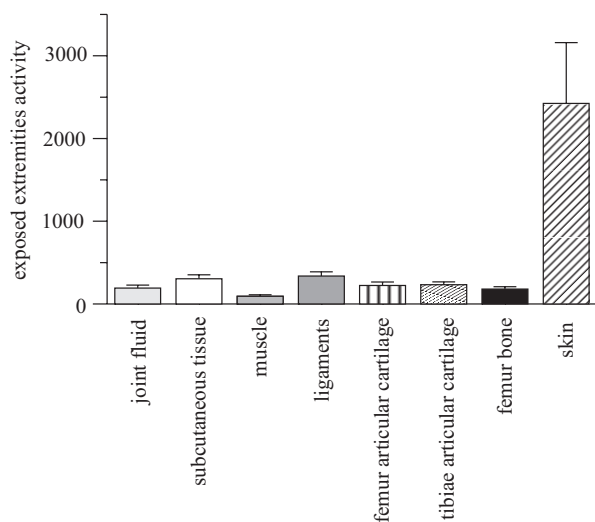


Fig. 3. Mean specific activity of the 48 hours-exposed extremities tissues expressed in counts per minute. Whiskers represent the standard errors (SEs) of the mean. The activity was normalized to the ointment activity of 1 MBq.

Vertical lines represent the standard error of the mean. High values found in skin samples may be partly due to incomplete absorption of active ingredients and the residuals of the ointment on the skin.

Mean values (\pm standard error) of extremities; indexes are presented in Fig. 4.

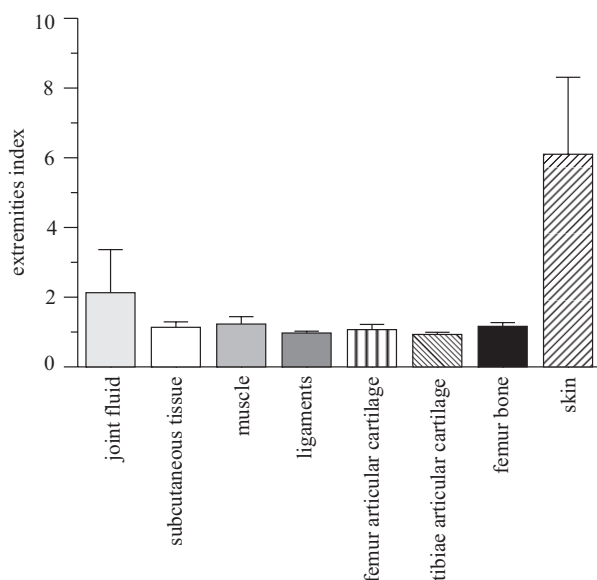


Fig. 4. Mean values of extremity indexes in the examined rabbits. Vertical lines represent the standard errors of the mean (SEM).

Whiskers represent the standard error of the mean. The highest values have been found in the skin, and the differences between *tissue* activities of ex-

posed and unexposed extremities were statistically insignificant (except in the skin).

The mean tissue/blood ratio of activity in the liver, kidneys, heart, lungs, and thyroid gland is presented in Fig. 5.

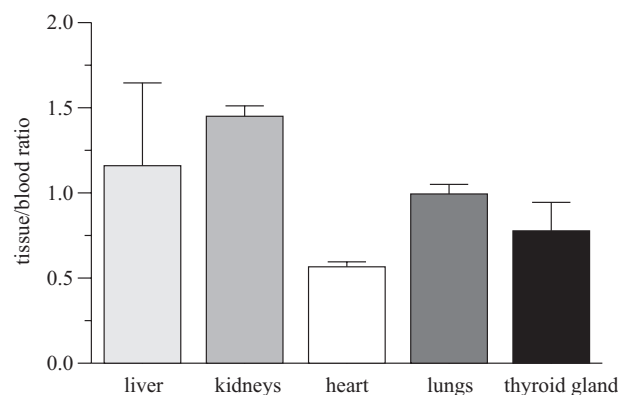


Fig. 5. Mean values of tissue/blood activity ratios of the exposed rabbits. Whiskers represent SEM.

As previously mentioned, the whiskers represent the standard error of the mean.

Statistically significant correlation was found between the activity of blood and kidneys (correlation coefficient $r = 0.9555$), blood and heart ($r = 0.9219$), blood and lungs ($r = 0.9218$), and blood and liver ($r = 0.9476$).

Histopathological investigations

Tissue samples taken from the control group and exposed rabbits underwent histopathological examination. Abnormal findings were found in all the investigated organs: edema and hyperemia in the lungs; muscle undulatio fibrarum, fragmentatio fibrarum, and hyperemia in the heart; and hyperemia in the kidneys and the liver. Similar results were also found in the respective investigated organ in the control rabbits (Fig. 6, Fig. 7, Fig. 8, Fig. 9).

Similar histopathological results were observed in rabbits exposed to the radioactive compound and those unexposed. These findings can confirm the thesis that the radioactive compound, labeled with I-131 Phlogahyl ointment, is not harmful to rabbit organs. This means that labeling did not influence the pharmacodynamics of the substance. Abnormal findings seen in histopathological samples are typical results observed in animals euthanized with Morbital. Histopathological studies excluded the pathological changes in all groups of rabbits.

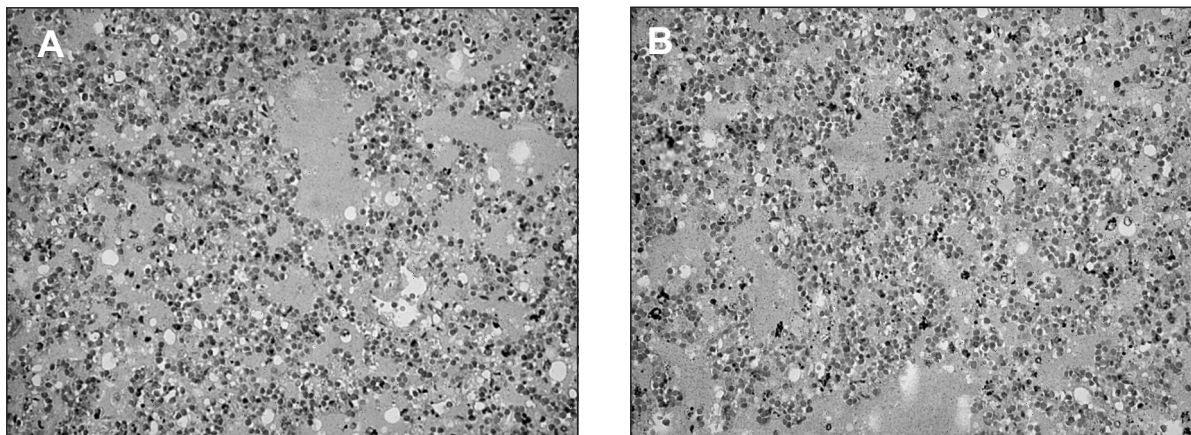


Fig. 6. Histopathological findings in the lung of control (A) and exposed rabbit (B) (H-E staining; magnification, $\times 200$). Edema and hyperemia are observed in both cases.

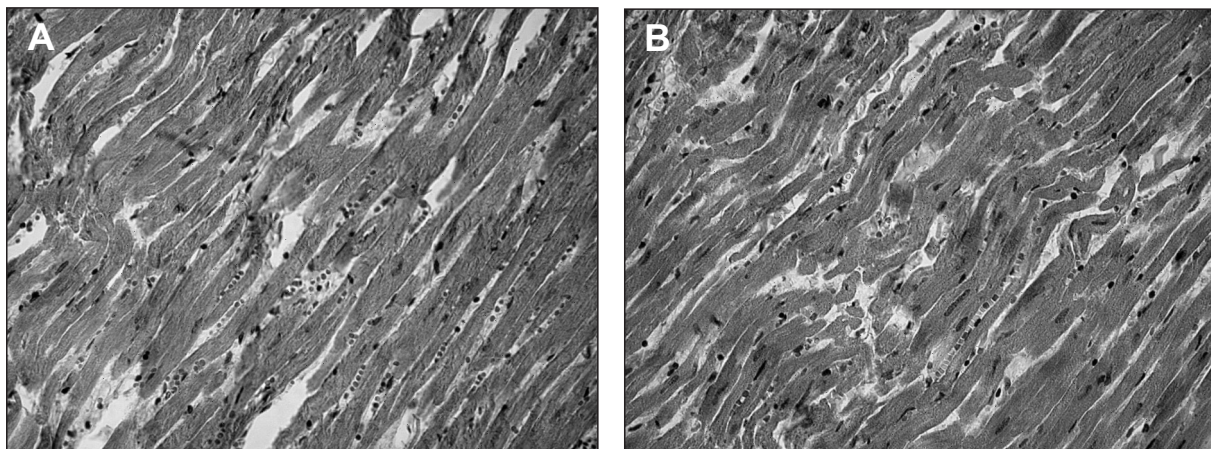


Fig. 7. Heart tissue findings in the control (A) and exposed rabbit (B) (H-E staining; magnification, $\times 200$). Hyperemia and undulation fibrarum are seen in both cases.

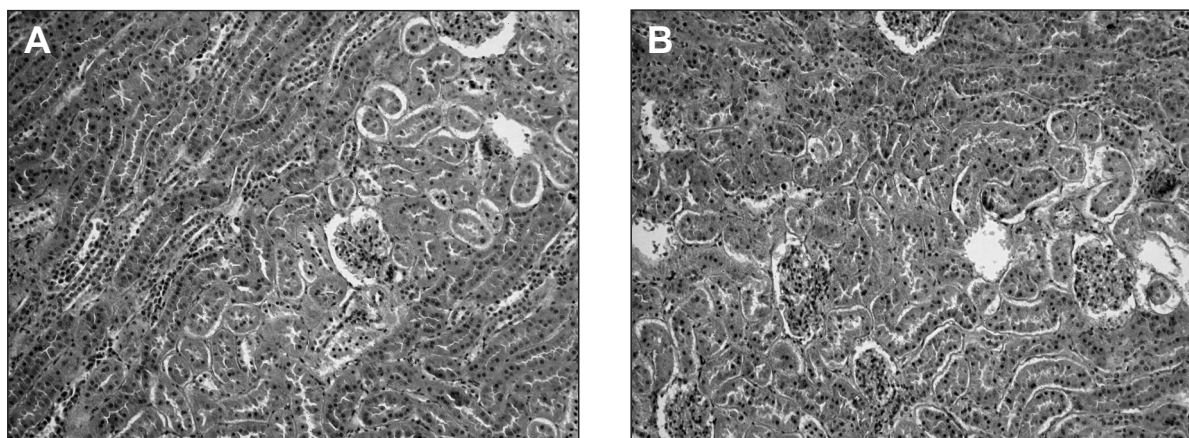


Fig. 8. Histopathological findings in the kidney of control (A) and exposed rabbit (B) (H-E staining; magnification, $\times 200$). Hyperemia is found in both cases.

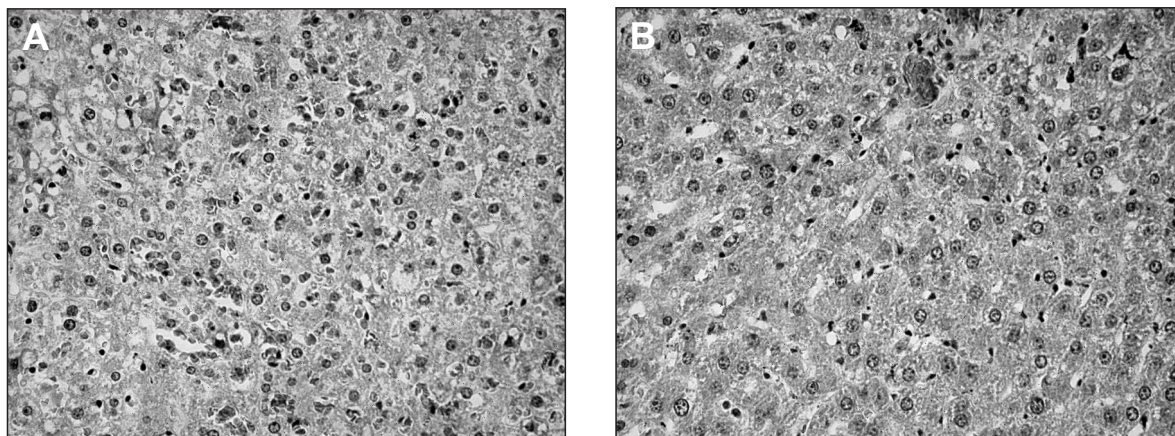


Fig. 9. Histopathological findings in the liver of control (A) and exposed rabbit (B). Similar picture of liver hyperemia is seen in both cases.

Discussion

To obtain successful therapeutic action, the proper drug and/or its active metabolite concentration in the target tissue should be achieved. The observed concentration will depend on the route of administration and the schedule of treatment. Most drugs are administered systemically, whereas in certain cases, the topical treatment seems to be beneficial. The advantage of such route can be partly due to the avoidance of first-pass metabolism. However, only a fraction of the drug applied to the skin surface reaches the systemic blood circulation and this amount will not necessarily reflect the concentration in the target organ (Klimowicz et al. 2007).

Majority of the compounds applied to the skin penetrate it by diffusion. Several factors can influence this process, including physicochemical properties, that is, molecular mass and shape, hydrophilicity and lipophilicity, temperature, blood flow, skin metabolism, and physiological and pathological conditions of the skin (Bos et al. 2000, Bielecka-Grzela et al. 2002).

As previously mentioned, several methods have been elaborated to investigate the penetration into the tissues. The application of labeled compounds is one of the techniques used for this purpose. In the present work, the penetration into various tissues of topical Phlogahyl ointment labeled with I-131 was investigated.

The efficacy of topically applied HA in the treatment of acute and chronic inflammation was investigated by others. Santalgelo (2007) performed an *in vitro* study on the effect of hyaluronan treatment on lipopolysaccharide-challenged fibroblast-like synovial cells that support the pharmacologic benefits of the treatment with HA. It can act by reducing the cellular interaction, binding the mitogen-enhancing factors,

or suppressing the production of proinflammatory mediators.

Kuemmerle (2006) reported a case of severe acute inflammatory reaction several hours after the intra-articular HA injection in horses. It required systemic nonsteroidal anti-inflammatory treatment and intra-articular steroid injection to solve the problem.

Bergin (2006) described the effectiveness of HA administered orally after arthroscopic surgery in 48 yearlings diagnosed with osteochondritis dissecans of the tarsus. Twenty-four horses were given 100 mg of HA orally for 30 days postoperatively, and the other 24 were given placebo. Experiment provided objective evidence that orally given HA reduces joint effusion.

In the present investigation, the good penetration of topically applied hyaluronate in the form of Phlogahyl ointment via the skin was observed. The concentration of HA in the subcutaneous tissue, muscle, ligaments, femoral articular cartilage, tibial articular cartilage and femur bone was similar in exposed and unexposed (symmetrical) extremities, whereas that in the joint fluid in the exposed extremity was slightly higher; however, the differences were statistically insignificant. Significant differences between the activity in exposed and unexposed extremity skin can be explained by the residue of the drug on the exposed extremity, nonabsorbed during the time of exposure.

The results of the study seem to explain the mechanism of drug delivery to the target tissue after topical application to the skin. As expected, no higher activity was found in the exposed leg tissues compared with the unexposed ones, showing that, after topical administration, the active components of the ointment are delivered to the joint via the blood stream. This route of HA administration seems to be useful for this drug delivery and can avoid unnecessary side effects.

Similar scheme of treatment, based on the topical application of the active components in form of the transdermal therapeutic system (TTS) was developed and is frequently applied (i.e. estrogens, testosterone, clonidine, nitroglycerin, scopolamine, fentanyl, nicotine). The drug applied topically, after absorption through the skin into the bloodstream is distributed in the body, penetrates into target tissue and exerts its therapeutic action. This route of administration seems to be useful, because the applied agent avoids the first-pass metabolism.

In conclusion, Phlogahyl ointment applied topically penetrates via the skin into the rabbits tissues and into the joint fluid of both legs (both exposed and unexposed) and does not achieve statistically relevant higher concentration in the joint of the exposed leg. The histopathological data did not show any side effects due to labeled Phlogahyl application. Considering the assumption that the labeled HA can mimic the behavior of other Phlogahyl ingredients, the results of this investigation suggest the utility and safety of topical application of this drug.

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