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MEDICINE

PHARMACOLOGICAL INFLUENCE OF ION CHANNEL BLOCKERS IN WOUND HEALING

PHD THESIS SUMMARY

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Introduction

Working hypothesis and general objectives

Skin wounds are definitely a problem of great medical interest given the high frequency with which they can be encountered in therapeutic practice, from traumatic injuries to surgical injuries. Their healing is generally a relatively long process even if they do not suffer complications and take many days, sometimes even weeks to heal.

The present work started from some literature data Veronica J. Moulin and Colab [1.2] which shows that at the level of skin wounds the electrical potential of cells at the edges of the wound is constant, while the potential inside the wounds is variable. This causes a potential difference between the edges and the inside of the wound to occur that varies throughout the healing process. This difference in potential can cause the cells to migrate into the electric field thus creating what could be important in the healing process of skin wounds [1–4].

The cells certainly migrate into the electric field due to the fact that they have a certain membrane electrical potential. This electrical potential is due to the migration of ions through the corresponding ion channels, which generates transmembrane electrical currents, respectively, depolarizing currents through sodium channels and calcium channels and repolarizing currents through potassium channels [1.2,5–7].

The blocking of the ion channels diminishes the intensity of these transmembrane ion currents, which is likely to alter the cell's electrical membrane potential and influence the speed at which they migrate into the electric field and, consequently, the duration of the wound healing process.

General methodology of research

The studies presented in this lcrare used practically only one research method, namely the assessment of the evolution over time of some standard lesions produced in rats.

Animals.

It was worked on male Wistar rats with an initial weight of between 230 and 300 grams. The rats were acquired INCDM (National Institute for Medical-Military Research and Development) Cantacuzino. The animals were brought to the Laboratory of the discipline of Pharmacology within the Faculty of Medicine of the University of Medicine and Pharmacy Carol Davila, at least 48 hours before the beginning of the experiment and were kept during the

entire experiment under standard conditions, namely food and water ad libitum, ambient temperature 21-23º C, artificial lighting 12 hours/day (8.00-20.00).

The rats were accommodated one by one in the cage and had access to water and food ad libitumla throughout the experiment. Also, the environmental conditions were constant throughout the experiment (brightness, temperature, humidity). The total number of rats worked was 196 and were divided into lots of 8-10 animals per lot, depending on the experiment.

All the experiments were carried out in compliance with the ethical norms laid down in the European Directive 63/2010 and Law 206 of 27 May 2004. All protocols were approved by the Ethics Commission of Scientific Research of the University of Medicine and Pharmacy Carol Davila and by the National Veterinary and Animal Safety Agency (ANSVSA), and the experiments were conducted under the supervision of the Animal Welfare Commission of the U.M.F. Carol Davila.

Substances

The substances used were:

Amiodarone – amiodarone hydrochloride hameln 50 mg/ml concentrate for solution for injection/infusion, SC. hameln rds s.r.o

Nimodipina - Nimotop 10 mg/50 ml soluție perfuzabilă SC BAYER AG

Ketamine 50mg/ml solution for injection PANPHARMA GmbH

Xylazine 2% solution for injection, BIOVETA

Alcool benzilic – solventul amiodaronei, LABBOX

Ethyl alcohol – nimodipine solvent, Prodvinalco

Distilled water

Saline

Betadine

They have performed successive dilutions, the concentrations of the solutions used being the following:

- For amiodarona: 200 000 nM
  2000 nM
  200 nM
- For nimodipina: 10 000 nM
  1000 nM

200 nM

In experiment number 1 and 2, the 3 concentrations of amiodarone and benzyl alcohol were administered compared to a batch that received nothing. In experiment number 3, the 3 concentrations of nimodipine and benzyl alcohol were administered, compared to a batch that received nothing. In experiment number 4, amiodarone was administered in a concentration of 200 Nm, nimodipine in a concentration of 2000 Nm and ethyl alcohol, compatible with a batch that received nothing. In experiment number 5, amiodarone was administered in a concentration of 200 Nm, nimodipine in a concentration of 2000 Nm administered simultaneously and sequentially, compared to a control lot.

Equipment

The equipment used was:

Syringes of 2ml, 5ml and 10 ml

Pipeta monocanal Isolab

100ml glass vials

Disposable surgical scalpel type number 11

Veterinary biopsy dermatom punch biopsy type, Kai Industries Co. ITD

Cardboard templates

Comprese sterile

Black marker

Disposable shaver

Pensa Chirurgicală Tissue 3:4, 14 Cm

Canon DSLR camera (3X optical zoom; 24.1 megapixels)

Laptop Dell cu Windows 10 si Office 10

Working mode

5 successive experiments were conducted.

On day 0 of each experiment, the rats were anesthetized with a cocktail of ketamine 100 mg/kg and xylazine 2% as follows: Each rat was injected with 91 mg/kgc ketamine and 9.1 mg/kgc xylazine.

After the general anesthesia was installed, the rat was placed on the working table and had a portion of the skin removed from the dorsal area – about 1 cm from the scapula. The

epilation was done as follows: First the rat's fur was trimmed with a scissors and then with the help of disposable razors - one for each rat - the remaining hair was removed from the dorsal area chosen for the lesion. The epilated surface was approximately 4 x 4 cm.

In the first experiment in the middle of the epilated area, a piece of cardboard was placed (a square - 1 x 1cm) and its outline was drawn using a black marker. Then the entire epilated area was disinfected with betadine.

With the help of a scalpel, an incision was made in the entire thickness of the skin (up to the panicle carnosus) along the contour drawn with the marker. With the help of a pen the skin was removed from the marked area. The area of the lesion obtained by excision of the skin was disinfected with betadine. Then the rat was placed in the cage. Full recovery from anesthesia was achieved after about 30 minutes [8–10].

In the following experiments to decrease the standard deviation, starting with the following experiments, instead of the 10 mm square lesion with the scalpel as we did in experiment number 1, I have produced skin lesions with the help of a punch biopsy veterinary biopsy dermatoma produced by Kai Industries Co. ITD, which produces standard circular lesions with a diameter of 8 millimeters. In experiment number 2, 3 lesions occurred in each animal, namely one in the head, one in the dorsal and one in the caudal. In experiments 3, 4 and 5 only dorsal and caudal lesions were produced without lesions in the head [11].

The next day (day 1 of each experiment) measured the surface of each lesion in each rat (an example is shown in Figure 1)

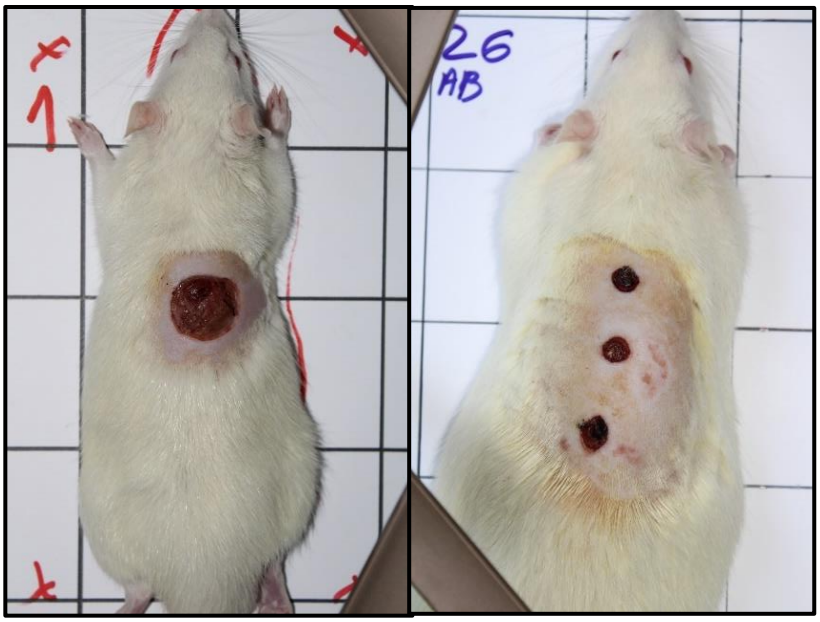

Figure No 1. Types of injuries performed on the first day of the experiment

Based on these measurements, the animals were divided into batches of 8-10 animals per batch, depending on the experiment, so that the average area and standard deviations were as close as possible between batches.

The test substances were administered starting from day 1 of the experiment (day 2 post-tegumentara excision), during the first 3 days the inflammatory stage of the healing process took place. From day 4 post-excision begins the proliferation stage and the administration of substances from this day followed the analysis of the effect of the substances to be investigated on the proliferative phase of scarring. The substances were administered using 5 ml syringes (without needle). Administration of the substances was carried out 2 times a day (morning at 7:00 a.m. and evening at 6 p.m.) through local applications – 2.5 to 3 ml/lesion/application so that the entire surface of the lesion is completely and relatively evenly covered. The substance was administered until day 15 (inclusive) except for the last experiment in which the wounds healed within 12 days [8.11].

The animals were followed until complete healing. It was also assessed the weight variation of rats by weighing them every 2 to 2 days. Each animal measured the surface area of each lesion on days 1.3, 5, 7, 12 and 15 [8.11].

To measure the area of the lesion, each rat was placed on a piece of cardboard (on which

various landmarks were drawn – 5 x 5 cm squares) and several landmarks of the rat position were marked (landmarks that were the same for the following measurements). For each rat, a cardboard was used to record the rat number. At a height of 40 cm from the back area, a Canon DSLR camera (3X optical zoom; 24,1 megapixeli). The same device settings and conditions were used to take all the photos (same height, same rat position – with the lesion located in the center of the image, ruler placed next to the rat, optical zoom) , respectively at the time t1- day 1 from the practice of the lesion, t2- day 3, t3 - day 5, t4 - day 7, t5 - day 9, t6 - day 12, t7 - day 15 [8.11]. At the end of the experiment, the rats were euthanized with a diazepam and ketamine cocktail.

The area measurement was performed using the ImageJ program. For scaling, the number of pixels/cm has been measured under the shooting conditions mentioned above A scale of 300 pixels/cm was used and a 50% zoom was used for all measurements of the lesion area. For each lesion, 5 measurements were made to minimize the human error because the area of the lesion was demarcated manually. The final size of the lesion area was the average of the 5 measurements.

Processing of results

The main parameter analyzed was the mean duration of wound healing in each batch, in the first experiment, or 3 secondary parameters at the next experiments.

In addition to this, 2 secondary parameters were analyzed, namely the percentage decrease of the lesion surfaces and the average speed percentage per day of the lesion surfaces [8.11].

For each rat and time of measurement, the following parameters were calculated:

a) The main parameter - the duration of wound healing measured in days

b) Secondary parameters

1. The percentage decrease of the area relative to the initial area value, according to the formula

$$S = \frac{S_{t1} - S_t}{S_{t1}} x100$$

Where S represents the percentage decrease in the measured area in percentage, St_1 represents the initial area measured in pixels, and St represents the area at the time of measurement in pixels.

2. The daily percentage decrease rate of the lesion surface according to the formula

$$V = \left(\frac{S_t - S_{t+1}}{S_t} x100\right) : [(t+1) - t]$$

Where V represents the daily percentage decrease rate of the area measured in %/day, St represents the area of the lesion at time t, measured in pixels, S_{t+1} represents the area of the lesion at time t+1 measured in pixels, and t represents the time of the area measurement expressed in number of days since the onset of the experiment.

3. The area under the curve of the evolution of the lesion surface over time after the formula

$$ASC = \sum_{t=1}^{6} (St_x + St_{x+n}) \frac{(t_{x+1} - t_x)}{2}$$

Where ASC represents the area under the curve of the lesion surface evolution in time measured in pixels x days, St_x represents the area measured in pixels at the time of tx, and txrepresents the moment of measurement in days.

Microsoft Excel was used to process the results. For each batch of experiments, the standard averages and deviations were calculated at each parameter at each moment of the measurement. The statistical significance of the difens for each experiment was investigated by the T-student test. The differences between the lots for each moment of the measurement were considered to be statistically significant if p <0.05 for the main parameter. For secondary parameters the Bonferroni method was applied in order not to produce alpha risk inflation [8.11].

Basically, the value of 0.05 was divided by the number of secondary parameters plus the number 1 from secondary parameters and it was considered that the differences between the lots are statistically significant if p is less than the value resulting from this division. In fact, for secondary parameters it was considered that the differences were statistically significant if p 0.02 for the first experiment in which there were only 2 secondary parameters and p 0.01 in the following experiments in which there were 3 secondary parameters [8.11].

Results

To verify this hypothesis, *in a first experiment* he studied the influence of the healing process of wounds with an area of 100 mm2 by amiodarone in 3 concentrations, respectively a concentration of 200 Nm that blocks only the potassium channels, a concentration of 2000 Nm blocking the potassium channels as well as the calcium channels and a concentration of 200 000 Nm blocking the potassium channels, calcium channels and sodium channels.

As regards wound healing (main parameter), only amiodarone in the low dose recorded a statistically significant decrease compared to the untreated batch, respectively 19.57 days in amiodarone in low concentration compared to 27.17 days in the untreated batch [8].

In principle, amiodarone in the low dose decreased the duration necessary to heal wounds by about 8 days, representing 27.97% of the time needed to heal wounds in the untreated batch.

Given the working hypothesis, we can assume that blocking potassium channels accelerates wound healing in our experimental conditions, represented by decreasing the time needed to heal wounds.

This assumes that blocking potassium channels promotes wound healing, taking into account that the average dose that besides potassium channels blocks calcium channels and high dose, which besides potassium channels also blocks calcium and sodium channels, has not had a statistically significant effect, we can assume that both the blocking of calcium channels and the blocking of sodium channels antagonize the effect of blocking potassium channels [8].

The hypothesis seems to be logical because the potassium current is a repolarizing current, and the calcium and sodium currents are depolarizing currents. It is very likely that the blocking of the potassium current that is repolarizing increased the membrane potential and thus accelerated the migration of cells into the electric field. Under these conditions, the blocking of depolarizing currents decreased the increased membrane potential by blocking the potassium channels and thus antagonized the favorable effect of blocking the potassium channels. Indeed, this seems to have happened in our experiment, because the effect of amiodarone in high concentration on wound healing was less intense than the effect of amiodarone in medium concentration. The differences are not statistically significant.

Secondary parameters used, respectively the percentage decrease of the lesion surfaces relative to the initial surface and the daily percentage decrease rate of the lesion surfaces try to assess by which mechanisms the blocking of the ion channels influenced wound healing.

As for the lesion surface, it gradually decreased from one determination to another for all batches. The fastest decrease was for the group treated with amiodarone in low concentration, the differences being statistically significant compared to the untreated group at all determinations, except for the time t4when no statistically significant differences were recorded from the control group. Amiodarone in high concentration also recorded statistically significant differences from the control group, but only at the time of t_3.

In terms of the daily rate of decrease of the lesion surface, it generally increased during the first 7 days, after which it remained relatively constant. The highest daily surface decrease rate was found in the group treated with amiodarone in low concentration, the differences being statistically significant compared to the batch not treated at t_2.

And the batches treated with amiodarone in medium and high concentration recorded higher daily percentage decrease rates of the injury surfaces than the untreated batch, but only the average dose of amiodarone was statistically significant at the t_3 time.

These results show that shortening wound healing time occurs by accelerating the rate of decrease of the lesion surfaces.

The fact that the differences were statistically significant only at certain moments of the measurement, not at all, suggests that different mechanisms intervene during the healing process from one stage to the next, and that the blockage of potassium channels may favor wound healing only at certain stages of the process, depending on the mechanism involved in the respective stages. The idea of different mechanisms from one stage to the next in the wound healing process is also reinforced by the fact that in the first 7 days of the healing process, the average daily percentage speed increased from one determination to another, and subsequently it remained relatively constant. The fact that in the analysis of these secondary and amiodarone parameters in average concentration had statistically significant effects at certain points in the measurement, but amiodarone in high concentration never had statistically significant effects suggests that the blockage of calcium channels only partially antagonizes the effect of blocking potassium channels. The effect of blocking potassium channels appears to be completely antagonized only by concomitant blocking of both calcium and sodium channels.

From a statistical point of view, there is of course the possibility that the small number of lesions per group of animals did not give sufficient statistical power to show differences between the batches treated with different doses of amiodarone.

In these conditions , *the second experiment was performed* in which the number of injuries per lot was increased. Practically every rat was performed 3 dorsal lesions with surfaces of 50.24 mm2.

As for the average wound healing duration, the main parameter, amiodarone in low concentration decreased this duration statistically significantly both in statistical analysis of all lesions and in stratified analysis. The results are consistent with those of the previous experiment. The decrease was 2.17 days in the analysis of all lesions, 2.87 days for analysis of cumulative dorsal and caudal lesions and 2.09 days for neck injuries. Apparently, this shortening of the time needed for healing is less than that found in the previous experiment in which the shortening was 7.4 days. This is certainly due to the fact that the lesions in the previous experiment were much larger than the lesions in this experiment, that is, 100mm2in the previous experiment and 50.24 mm2in the current experiment. Naturally, larger lesions need a longer time to heal than smaller lesions. Specifically, the lesions practiced in the previous experiment in the control group were healed within a period of 27.17 days, and the lesions in this experiment were healed in the control group within a period of 15.83 days [11].

The overall lesion analysis did not reveal any different data than the previous experiment. This is probably due to a large dispersion due to the fact that the neck injuries had a different evolution from the dorsal and caudal lesions. The stratified analysis showed that in case of neck injuries there were statistically significant differences from the control group, in the batches treated with amiodarone in low and medium concentration. Switching to caudal and dorsal lesions there were statistically significant differences in the batches treated with amiodarone in low and high concentration.

This data confirms the hypothesis that blocking calcium channels and blocking sodium channels do not completely antagonize the blockage of potassium channels, but only diminish its intensity.

Secondary parameters used, namely the percentage decrease of the lesion surfaces relative to the initial surface, the daily percentage decrease rate of the lesion surfaces and the area below the evolution curve of the lesion surface over time try to assess by which mechanisms the

blockage of the ion channels influenced wound healing.

As for the decrease in the lesion surfaces, they decreased by one percent of the initial area progressively increasing from one moment of measurement to another. The reduction of the lesion surfaces was highest in the low concentration groups treated with aiodarone, but no statistically significant differences could be recorded from the control group until t_6.

Analysis of the evolution of the lesion surfaces of the cumulative dorsal and caudal lesions showed the same as above, the greatest decrease in the lesion surfaces was recorded at the low dose of amiodarone, the differences being statistically significant compared to the untreated batch at t_5 and t_6 [11]

In contrast to the first experiment, in the case of dorsal and caudal lesions and amiodarone in the mean concentration had a statistically significantly better evolution than the control group, except at t_4.

Amiodarone in high concentration also caused a better evolution of lesions than in the control group, but the differences were statistically significant only in the last period of their evolution, starting with t_5.

The statistical analysis of the evolution of neck injuries did not show practically statistically significant differences.

In terms of the average percentage rate of daily decrease of the lesion surfaces, similar to those found in the previous experiment, it increased progressively in the first 7 days, after which it remained relatively constant. The analysis on all lesions, however, showed that there were only some moments when statistically significant differences were recorded from the control group, at each of the 3 concentrations used.

As for the area below the evolution curve of the lesion surface over time in all cumulative lesions, they decreased statistically significantly compared to the control group at all doses of amiodarone, the largest decrease being recorded in the group treated with low amiodarone concentration. The statified analysis did not provide additional information on both cumulative caudal and dorsal lesions and for neck injuries, the amiodarone batches did not show statistically significant differences.

These data show that the shortening of wound healing time is due to the acceleration of wound healing speed, but that, at least apparently, blocking different types of ion channels only takes effect at certain periods of the healing process. At least apparently, based on the above

results, blocking potassium channels favors healing at certain times, blocking calcium channels at other times, and blocking sodium channels at other times.

Since the simultaneous blocking of potassium and calcium channels significantly shortens the duration of wound healing, the question has been raised whether the exclusive blocking of calcium channels influences wound healing.

Since blocking calcium channels along with blocking potassium channels also promotes wound healing, the question has been raised whether blocking only calcium channels has such an effect. Thus, *the third experiment* was done, where the rats were subjected to lesions with an area of 50.24 mm2, then were treated with nimodipine in 3 concentrations, a concentration of 200nm that blocks only the L-type calcium channels, and the results of the study were not completely different, a concentration of 1000 Nm blocking both type L calcium channels and type T calcium channels and a concentration of 10 000 nm blocking type L calcium channels, type T calcium channels and CB1 cannabinoid receptors.

As for the duration of the wound healing, the main parameter, nimodipine in average concentration decreased this duration from 17.33±6.37 days in the control group, up to 14.8±5.6 days, that is, 2.53 days. If we make a comparison regarding the shortening of the duration of wound healing in the 3 experiments presented so far the situation is presented as follows:

- In the case of large lesions in the first experiment, the duration necessary for wound healing from 27.17±5.19 days to 19.57±3.05 days, that is, reduced by 7.6 days, which represents a 27.97% reduction.

- In the case of small lesions, the low concentration amiodarone reduced the duration of wound healing from 17.37±2.19 days to 14.50±2.19 days, that is, reduced by 2.87 days, which is a 16.52% reduction.

- In the case of small lesions treated with nimodipine, the average dose of nimodipine reduced the duration of wound healing from 17.33±6.37 days to 14.8±5.6 days, i.e. reduced by 2.53 days, which is a 14.59% reduction.

These data suggest that blocking potassium channels promotes wound healing, the more the larger the surface. When the lesions had an area of 100mm2reduction in wound healing time was 27.97%, and when the lesions were 50.24mm2, the reduction in wound healing time was 16.52%.

And blocking of calcium channels favors wound healing under our experimental conditions, this only happens by simultaneously blocking the L-type calcium channels and T-type calcium channels, because only the average nimodipine had a statistically significant effect. Low-concentration Nimodipine, which only blocks L-type calcium channels, had no statistically significant effect. Of course, it is difficult to say whether the favorable effect found at the average dose of nimodipine is due to the exclusive blocking of T-type calcium channels or the concomitant blocking of both types of calcium channels. However, the fact that selective blocking of L-type calcium channels did not have a statistically significant effect makes us believe that the effect found is due exclusively to blocking of T-type calcium channels

Whereas in the case of blockage of potassium channels, on small-scale lesions, the decrease in the duration required for wound healing was 16.52%, and in the case of blockage of calcium channels of type T the decrease in this duration was 14.59%, it suggests that the effect of blocking potassium channels is more intense than blocking calcium channels of type T.

Secondary parameters used, namely the percentage decrease of the lesion surfaces relative to the initial surface, the daily percentage decrease rate of the lesionelected surfaces the area below the evolution curve of the lesion surface over time tries to assess by which mechanisms the blocking of the ion channels influenced wound healing.

As regards the secondary parameters analyzed, only the daily percentage decrease rate recorded a statistically significant difference at t_3 for the batches treated with nimodipine in medium and high concentration.

The *fourth experiment*, given that nimodarone also favors wound healing, not only blocking the potassium channels exclusively through amiodarone, the question has been raised whether the combination of low-dose amiodarone with middose nimodipine favorably influences wound healing. They also worked on rats who were given 2 lesions of 50.24 mm2.

All the substances used shortened the wound healing time statistically significantly compared to the control group. The most intense effect was the blocking of potassium channels by amiodarone in the low dose, when the shortening of the wound healing time was 1.94 days, that is, a shortening of 13.06%. And the simultaneous blocking of L-type and T-type calcium channels by nimodipine in the average dose shortened wound healing time by only 0.18 days, that is, a shortening of 1.21%. The association between amiodarone and nimodipine decreased wound healing time by 1.99 days, or 13.4%, a value virtually identical to that produced by low-

dose amiodarone by blocking potassium channels.

It is obvious that concomitant blocking of potassium channels, L-type calcium channels and T-type calcium channels does not bring any significant benefit to the exclusive blocking of potassium channels.

From the previous experiment it was found that only blocking the T-type calcium channels favors wound healing, not blocking the L-type calcium channels

Corroborating the data from all the experiments presented so far, it is possible, as shown in experiment number 1, that the blocking of calcium channels of type L to partially antagonize the blocking of potassium channels, which probably does not cause the blocking of calcium channels of type T. this would really explain why no summation effect was found. Part of the effect of blocking potassium channels was antagonized by blocking the L-type calcium channels to which the favorable effect of blocking the T-type calcium channels was added

The analysis of secondary parameters showed that the duration of shorter wound healing time was produced by increasing the daily percentage rate of wound evolution with the mention that in the last stage of wound healing this speed was statistically significantly increased only by blocking the potassium channels through administration of amiodarone in low dose.

In terms of percentage decrease of the lesion surfaces, it was statistically significantly increased depending on the period of evolution of the lesions; In the first part of the healing process there were statistically significant deferences from the control group called by blocking the T-type calcium channels, only by administering nimodipine, while in the second part of the wound evolution there were statistically significant differences from the control group only by blocking the potassium channels by amiodarone administration.

It is possible that different functional mechanisms intervene in the implementation of this process in different stages of the wound healing process. Under these conditions, in the first phase of the wound healing process, some mechanisms could intervene that are accelerated by blocking the T-type calcium channels, and in the second part of the wound healing process, mechanisms that are accelerated by blocking the potassium channels could intervene.

In these circumstances *, the fifth experiment* was conducted in which the influence of the wound healing process by amiodarone was investigated in concentrations that block only the potassium channels and nimodipine in concentrations that block both the L-type calcium channels and the T-type calcium channels in sequential administration, i.e. nimodipine in the

first stage, followed by amiodarone in the second stage of wound healing compared to the nimodipine-amiodarone association in continuous administration throughout the wound healing process.

All substances caused a decrease in the healing time of statistically significant wounds, whether administered separately, concomitantly or sequentially. The decrease in wound healing duration was 11.38% for amiodarone, 12.61% for nimodipine, 13.07% for the combination of the two substances and 14.23% for sequential administration of the two substances. As can be seen, the greatest decrease in wound healing duration was indeed, according to the working hypothesis, in sequential administration of the two substances, but the differences between sequential administration and concomitant administration are not statistically significant.

The analysis of secondary parameters shows, as in previous experiments, that this shortening of the duration of wound healing is due to the increase in the daily percentage decrease rate of the lesion surface. However, this parameter was statistically significant only for sequential administration of substances in both nimodipine (t4 moment) and amiodarone (t5 moment). As regards the area below the curve of the lesion surface evolution over time, this parameter recorded statistically significant differences from the control lot only in the case of sequential administration of the two substances.

Under all these conditions we can appreciate that the practical working hypothesis is confirmed. Nimodipine acts in the first stage of the healing process by blocking the T-type calcium channels, while amiodarone acts in the second stage of the healing process by blocking the potassium channels. Analysis of the first two experiments revealed that the effect of blocking potassium channels is more intense as the surface of the lesions is larger. Specifically, on lesions with a surface of 100 mm2 the duration of wound healing was 27.97%, and on lesions with a surface of 50.24mm2 the duration of wound healing was shortened by 16.52%.

However, the data from this experiment showed that blocking potassium channels favors wound healing only in the second stage of this process when the average daily shortening speed remained relatively constant, not during the first period when the shortening speed increased progressively, the period that has practically the same duration of about 7 days regardless of the surface of the lesions. This second period lasted for 20.17 days in the case of 100 mm2 lesions and 8.83 days in the case of 50.24mm2 lesions.

In these conditions, in the case of large lesions, a decrease in wound healing duration of

7.4 days represents 37.67% of the duration of the second wound healing stage, and in the case of small lesions a decrease in wound healing duration of 2.17 days represents 24.57%.

Under these conditions, it follows that if the shortening of wound healing time is related to the period when amiodarone acts by blocking the potassium channels only, the effect is more intense as the surface of the lesions is larger.

The association of amiodarone to nimodipine partially antagonizes the effect of nimodipine in the first stage of wound healing, and the association of nimodipine to amiodarone partially antagonizes the effect of amiodaronia in the second stage of wound healing evolution, probably by blocking the L-type calcium channels

In our experimental conditions based on the above results, we can appreciate that the most effective way to use the above substances to speed up the wound healing process is the sequential administration, initially of nimodipine and later amiodarone. In addition to effectiveness, this method of treatment could also have the advantage of lower costs. The fact that the first stage of the wound healing process, namely that of pro-fat growth of the daily percentage rate, seems to be constant, around 7 days regardless of the surface of the lesions and the time needed to heal the wounds, allows to some extent a standardization of sequential therapy. According to the data obtained in our experimental conditions, nimodipine should be administered during the first 7 days of wound evolution, followed by amiodarone.

It is difficult to say whether this data obtained under our experimental conditions can be extrapolated to humans. It would also be ideal to conduct clinical trials.

Conclusions and personal contributions

Final conclusions

1. Amiodarone in low concentration, which blocks only the potassium channels, has caused the healing duration of the wounds to be shortened.
2. Medium-dose amiodarone, which blocks both potassium channels and high-dose calcium and amiodarone channels, which block potassium channels, calcium channels and sodium channels, did not result in a statistically significant shortening of wound healing duration.

3. In an experiment in which increasing the number of lesions per lot increased the strength of the test, it was found that amiodarone in medium concentration, which blocks both potassium channels and calcium channels, statistically significantly shortens the duration of wound healing, but the effect is less intense than that by blocking the potassium channels exclusively through a low concentration of amiodarone.
4. It is possible that the blocking of potassium channels that cause an increase in membrane potential, the potassium current being a repolarizing current, is partially antagonized by concomitant blocking of calcium channels, the calcium current being a depolarizing current.
5. The effect of blocking potassium channels on the duration of wound healing is more intense as the surface of the lesions is larger. In the case of lesions with an area of 100 mm2, the duration of wound healing was shortened by 27.97%, and in the case of lesions with an area of 50.24mm2, the duration of wound healing was shortened by 16.52%.
6. Nimodipine, in a low concentration that blocks only the L-type calcium channels, does not influence the duration of wound healing.
7. Nimodipine in medium concentration, which blocks both L-type calcium channels and T-type calcium channels, statistically significantly shortens wound healing time.
8. Blocking of T-type calcium channels has a favorable effect on wound healing.
9. The combination of nimodipine in the medium dose, which blocks both L-type calcium channels and T-type calcium channels, with low-dose amiodarone, which blocks only the potassium channels, does not cause a summation effect, although each of the 2 drugs administered separately significantly shortens wound healing.
10. Blocking only the L-type calcium channels does not promote wound healing, but it is possible to decrease the intensity of the effect of blocking the potassium channels.
11. Analysis of secondary explanatory parameters showed that nimodipine has statistically significant effects only in the first part of the wound healing period, while amiodarone has statistically significant effects only in the second part of the wound healing period.
12. The sequential administration of the two substances, namely nimodipine in the first stage and amiodarone in the second stage of the wound healing process, had a more

intense effect than the simultaneous administration of the two substances during the wound healing period.

13. It is possible that the favorable effect of blocking the T-type calcium channels by nimodipine in the first stage of the wound healing period is diminished by blocking the potassium channels by amiodarone.
14. Since the effect of nimodipine is due to the blocking of T-type calcium channels, it is possible that the amiodarone's damage is partially diminished by blocking the L-type calcium channels in the second stage of the wound healing process.
15. From the data presented above, it follows that the blockers of the ion channels influence the wound healing process.
16. This influence is likely due to the change in the electrical potential of some cells and the change in the speed at which they migrate into the electric field due to the difference in electrical potential that exists between the edges of the wound and the center of the wound.
17. Very likely in the first stage of wound healing, the migration of these cells is accelerated by decreasing the electrical potential of the cells as a result of blocking the T-type calcium channels
18. Also very likely, in the second stage of wound healing, the migration of cells into the electric field is accelerated by increasing the electrical potential of cells as a result of blocking the potassium channels.
19. The conclusions in paragraphs 17 and 18 agree with literature data showing that the electrical potential at the edges of the wound is constant, while the electrical potential inside the wound is variable during the wound healing process.
20. In our experimental conditions, the most effective way of treating skin wounds has been shown to be the sequential administration of nimodipine in the first stage, followed by amiodarone in the second stage of wound healing.
21. From the data obtained in our experimental conditions it follows that nimodipine should be administered during the first 7 days of treatment, followed later by the administration of amiodarone.

Personal contributions

1. We consider that the working hypothesis (chapter work hypothesis) according to which the blocking of the ion channels influences wound healing by influencing the membrane potential of cells migrating into an electric field whose existence has been demonstrated by other authors is original because it has not been found in the literature.
2. We believe that the above hypothesis was demonstrated by this paper because both the blocking of potassium channels and the blocking of calcium channels of type T shortened the healing time of wounds in our experimental conditions (Chapter 8, 9 and 10).
3. It is the first time in the literature that it has been shown that blocking the T-type calcium channels favors wound healing only in the first 7 days of this process, and blocking the potassium channels only at the later stage (Chapter 10).
4. This is the first time in the literature that amiodarone has been studied on wound healing (chapters 6, 7, 9 and 10).
5. It is the first time in the literature that amiodarone is studied in different concentrations that block different ion channels: Potassium channels, potassium and calcium channels, potassium, calcium and sodium channels (Chapter 6 and 7).
6. It is the first time in the literature that the effect of nimodipine on the healing of experimental wounds is studied; other calcium channel blockers have been studied) (chapter 8)
7. This is the first time in the literature that nimodipine is studied in different concentrations that block different ion channels, namely only L-type calcium channels or both L-type calcium channels and T-type calcium channels (Chapter 8).
8. It is the first time in the literature that it is shown that only blocking the T-type calcium channels promotes wound healing and not blocking the L-type calcium channels (Chapter 8, 9 and 10).
9. It is the first time in the literature that the administration of T-type calcium channel blockers by nimodipine (in the first part of the healing process) is studied, followed by blocking of potassium channels by amiodarone (in the second stage of the healing process) (chapter 10).

10. Our experimental research has shown for the first time in the literature that the most effective way to treat wounds by blocking ion channels is to administer sequentially, in the first 7 days, some T-type calcium channel blockers (in our case nimodipine). followed by blockage of potassium channels (in our case amiodarone) in the second period (chapter 10).
11. The results in point 10 were based on the observation that wound healing occurs in two distinct stages in the rat, that is, a first stage lasting about 7 days regardless of the time needed to heal the wounds followed by a second stage that begins on the 8 day and ends with the total healing of the wounds no matter how long this process is (chapter 6, 7, 9 and 10).
12. It is difficult to say whether this observation is extrapolable in humans; it is practically expected that the healing of wounds in humans will also occur in two stages, but we do not know how long the first stage is in clinical conditions (chapter 10).
13. We believe that clinical trials of human skin wounds would be required to extrapolate our results in humans (Chapter 10).
14. It is the first time in the literature that the hypothesis of clinical studies on the acceleration of wound healing by sequential topical administration of nimodipine and amiodarone is proposed (Chapter 10).

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List of scientific works

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