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СТАБИЛИЗАЦИЯ ПРОСТАГЛАНДИН *H*-СИНТЕТАЗЫ В ПЕКТИНОВЫХ ПЛЕНКАХ, ИСПОЛЬЗУЕМЫХ ДЛЯ ЛЕЧЕНИЯ КОЖНЫХ РАН

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Представлен новый метод иммобилизации ПГНС с использованием природного полимера пектина в качестве носителя; иммобилизованную таким образом ПГНС можно применять для лечения кожных ран. ПГНС выделяли из семенных пузырьков быка, проводили ее иммобилизацию на пектине, исследовали стабильность и активность. При хранении фермента при –15°С ферментативная активность сохранялась до 88% (через 5 дней) и до 41% через 60 дней. Эффективность иммобилизованной ПГНС в залечивании ран тестировали на мышах CD1, измеряя линейные размеры ран, обработанных и необработанных ПГНС. Обработка ПГНС ускоряла залечивание раны и стимулировала образование в ране простагландина E_2 ($ПГЕ_2$). Иммобилизованная ПГНС способствовала увеличению удельной скорости прогрессирования заживления раны (m*), которую рассчитывали как тангенс угла наклона прямой в полулогарифмических координатах. Предложена кинетическая схема процесса заживления раны в зависимости от концентрации ПГНС.

INTRODUCTION

Prostaglandin H synthase (PGHS, cyclooxygenase, COX, prostaglandin endoperoxide synthase, EC 1.14.99.1), a key regulatory enzyme in the biosynthetic pathway of prostaglandins, metabolizes arachidonic acid into prostaglandins H_2 (PGH_2) intermediates, which in turn serve as a precursor for the biosynthesis of 2-series prostaglandins, thrombonxanes and prostaciclin, known also as prostanoids [1]. Prostaglandin E_2 (PGE_2) and prostaciclin (PGI_2) have been shown to control angiogenesis and play an important role in wound healing in soft tissues, although the precise mechanism of action is still unknown [2, 3].

Previously we demonstrated that the PGHS enzyme application can markedly accelerate the process of skin repair in a mouse model as well as the orthodontic tooth movement in cats [4, 5]. So, this enzyme is undoubtedly of pharmacological interest, since it can be applied to regulate the level of a wide array of prostanoids. However, fast and irreversible inactivation of the enzyme in the course of catalysis [6] and upon storage [7] makes it difficult to use PGHS for enzyme therapy.

The storage stability of the enzyme can be increased by the addition of calcium ions [7] or by immobilization [8] that can be monitored though its activity. PGHS exhibits two enzymatic activity that can be measured by detecting of oxygen consumption (cyclooxygenase activity), and by monitoring of chromogenic electron donor oxidation (peroxidase activity).

Previously, it has been demonstrated that the enzyme is highly sensitive to the presence of components used both in the covalent chemical bonding of enzyme with a matrix applied to enzyme immobilization, and in the formation of gels using radical ions [9]. The enzymatic system of prostaglandin synthesis from sheep vesicular glands can be immobilized by adsorption on EAE Sephadex [9] and silica gel [8]. These materials are not suitable for cutaneous enzyme application.

The PGHS application for wound treatment requires an alternative immobilization technique. Films made from natural products are of increasing scientific and commercial interest; they are not only biodegradable, but may be acceptable for pharmaceutical applications. Previously, we reported that residues of passion fruit (*Passiflora edulis*, maracuya) is a good source of natural polymer (pectin) that has potential in the development of new materials for skin injury treatment based on entrapped papain [10]). This gel entrapment method is attractive because it is very simple and can be carried out under mild conditions (physiological, pH and tempera-

ture). In the test with subcutaneous injection of pectin solutions and pectin film application, there were no negative secondary effects observed on the mice skin and the process of wound repair [10]. The film can easily be applied to a surgical wound and be removed by washing (due to pectin solubility in water) without alteration of tissue that sometimes take place in the case of solid materials, for example gauze.

In this paper we report on: 1) the stability of PGHS from bovine vesicular glands during immobilization on pectin films and under storage at 4 and −15°C; 2) the effect of different activities of immobilized PGHS on the kinetics of wound healing and PGE_2 level; 3) the kinetic model to explain the effect of PGHS on wound repair and 4) the effect of presence of arachidonic acid on specific rate of progress of wound healing.

MATERIALS AND METHODS

Enzyme preparation

Microsomal fraction containing PGHS was isolated from bovine vesicular glands according to the method described previously [11], applying the capacity of calcium ions to precipitate microsomes by means of centrifugation at 10 000 g. The following reagents were purchased from Sigma-Aldrich (USA): Trizma, diethyldithiocarbamate sodium salt (ETC), Tween-20, $CaCl_2$, HCl, arachidonic acid, L-adrenaline, hemin. ETA disodium salt and ethanol were purchased from Baker Analyzed (USA).

Immobilization of microsomal PGHS by entrapment on pectin film

Pectin was extracted from the shells of passion fruit (Pasiflora edulis) acquired from the juice factory in Puebla, Mexico using the method reported previously [10]. The pectin films containing microsomal fraction with PGHS and without enzyme were prepared using 1.25% pectin solution in water in the presence and absence of glycerin at 0.7% (v/v) that was added as elasticizer to make the films sufficiently flexible. Viscous solution of pectin (0.3 ml) was poured directly into a plastic dishes followed by an addition of 0.2 ml of different dilutions of PGHS preparation or water (to prepare control pectin films without enzyme). Good reproducibility was attained by using fixed volumes for a uniform casting environment and the same volume of microsomal PGHS. The mixtures were air-dried for 24 h at room temperature (22–25°C). Each film was easily peeled from the plastic dish for use in the subsequent experiments.

PGHS activity measurement

The PGHS activity was determined as the rate of oxygen consumption in the cyclooxygenase reaction of arachidonic acid transformation by continuous oxygen detection using HANNA Instruments HI 964400. The mixture of immobilized microsomes (2.29 ml) or microsome solution (0.2 ml), 1.3 mM adrenaline, 0.003 mM hemin, and 0.19 mM arachidonic acid was placed in the tightly closed cuvette with stir bar and incubated at 25°C for 7 min. Measurement of oxygen consumption was performed during the incubation period. The rate of oxygen consumption was plotted as a function of time. The initial rate of reaction was determined as the tangent of the inclination angle of the initial part of the kinetic curve.

The enzyme activity was detected during immobilization and at different time of enzyme storage, as well as prior to film application on the skin wound.

Study of wound healing kinetics

The effect of pectin and immobilized PGHS on tissue repair was tested in C1 mice. All experiments were carried out using aseptic technique with regard to anesthesia and surgery. Six mice were used for the experiment. Three linear wounds (L_0 = 15.5–16.5 mm) were created on the back of each mouse. Pectin films with and without PGHS were applied on different wounds of the same mouse immediately after wounding. The third, control wound had no films. The linear size of each wound was recorded daily.

In a second experiment only one wound (L_0 = 15–17 mm) per mouse was created (5 groups of 6 mice were used), then pectin films with PGHS preparation of different activities were applied immediately . The lineal size of each wound was recorded daily Statistic was performed using nonpaired Student's t test. Lineal size determination of each wound was performed daily as a measure of progress of wound healing. A difference was considered significant if $P<0.05$.

HPLC measurement of PGE_2 in the wounds

This assay was performed in three steps: 1) wound creating and treatment application; 2) PGs extraction and 3) PGE_2 determination by HPLC.

Two wounds (1 cm^2) were created on the back of each mouse. Seven mice were used for this test. A pectin film (placebo) was applied on one wound and a pectin film with PGHS microsomal preparation was applied to the second wound. After 1 hour of therapy, the mice were sacrificed, the skin samples were immediately extracted, weighted, and prostaglandin extraction was performed.

The skin samples were divided into two groups: skin wounds treated with PGHS and skin wounds treated

with placebo. Each group was homogenized in 2 ml of phosphate buffer (pH 3.5), and then 2 ml of ethyl acetate pH 3 were added. The organic extracts were dried over Na_2SO_4 and evaporated to dryness under reduced pressure. The resulting samples were dissolved in 0.5 ml of acetonitrile and used for HPLC determination of PGE_2.

A HPLC system consisting of a model 1050 HEWLETT PACKAR with UV detector was operated at ambient temperature [12]. C18 column (4.6 mm×25 cm, Syn Chropack RP-P) and 35% acetonitrile in 2 mM phosphate buffer (pH 3.5) as a mobile phase were used for analysis The flow rate and injection volume were 1 ml/min and 0.01 ml, respectively. The retention time of PGE_2 that was of 5.935 +/− 0.2 minutes. The UV-detection was performed at 205 nm. Stock solutions of PGE_2 that was added to extracted samples to obtain calibration curve were prepared at concentration 2.56, 5.11, 10.22, and 25.6 nM. Pick areas were presented as the function of PGE_2 concentration. The PGE_2 concentration was determined as absolute value of the abscissa of intersection point of obtained line and concentration axis. The measurements were performed by duplicate.

Kinetic model

A kinetic model was proposed to describe of the specific rate of healing progression as the function of PGHS concentration applied for the treatment, considering the increase of PGE_2 concentration. The Monad's and Mechaelis−Menten equations were used to explain the wound healing progression related with the cell proliferation and the enzymatic synthesis of prostaglandin E_2.

Study of effect of arachidonic acid on the kinetics of wound healing

In this assay 2 groups of 6 mice each were used. One wound (L_0 = 16 mm) per mouse was created. To the wounds of the first group 0.04 ml of physiological solution were applied, mice in the second group were treated with 0.04 ml of 7 mM arachidonic acid in physiological solution. The healing of the wounds was determined by daily measurement of the linear size of the wounds as described previously.

RESULTS AND DISCUSSIONS

Immobilization of microsomal PGHS and its stability

The application of PGHS enzyme to a skin wound has been shown to accelerate the process of tissue repair [4]. To improve enzyme stability under preparation and storage conditions as well as to simplify the procedure for its introduction to the wound, we immobilized the enzyme on natural polymer pectin.

Pectin was chosen based the following properties: good solubility in water (unlike gauze, it can be easily removed by washing after treatment), atoxicity, the presence of exchange ionic groups, and lack of interference in the scaring process [10].

Pectin films were obtained in the presence or absence of glycerin, with a thickness of 0.025−0.040 mm. Glycerin at 0.07% (v/v) was used to improve the mechanical properties of the films. Resistance to mechanical stretching in the presence of glycerin was 6 times higher, although the addition of the enzyme affected the mechanical characteristics considerably [10].

Enzyme activity during immobilization (Table 1) and storage stability (Table 2) was studied. In the course of immobilization, we observed a decrease in enzyme activity since the films were dried at ambient temperature (Table 1). Immobilization, however, led to some enzyme stabilization in comparison with enzyme watery solution. The presence of glycerin had a negative effect on enzyme activity during immobilization and storage. In pectin film without glycerin enzyme lost 3−4% of its initial activity, while in the presence of glycerin, activity loss was 66% (Table 1). In all previous reports related to the stability of immobilized PGHS, the activity loss was

Table 1

PGHS activity during pectin film formation

Sample	Activity, O_2 ppm/sec	Relative activity, %
PGHS Water solution	0.0444+/–0.0031	100+/–7
PGHS + pectin solution before drying	0.0428+/–0.0024	96+/–5
PGHS + pectin + glycerin solution before drying	0.0304+/–0.0041	68+/–9
PGHS + pectin film (after 24 h of drying)	0.0431+/–0.0136	97+/–8
PGHS + pectin + glycerin film (after 24 h of drying)	0.0152+/–0.0032	34+/–7
PGHS Water solution (after 24 h of drying)	0.0062+/–0.0011	14+/–2

Table 2

Storage stability of immobilized PGHS at 4 and –15°C

Days of storage	PGHS on pectin film				PGHS on pectin film with glycerin			
	4°C		–15°C		4°C		–15°C	
	Activity, O_2 ppm/sec	Relative activity, %	Activity, O_2 ppm/sec	Relative activity, %	Activity, O_2 ppm/sec	Relative activity,%	Activity, O_2 ppm/sec	Relative activity, %
0	0.0431+/–0.0136	100	0.0431+/–0.0136	100	0.0152+/–0.0032	100	0.0152+/–0.0032	100
5	0.0147+/–0.0020	34	0.0379+/–0.0040	88	0.0039+/–0.0005	26	0.0145+/–0.0095	95
8	0.0190+/–0.0031	44	0.0278+/–0.0031	64	0.0017+/–0.0005	11	0.0019+/–0.0003	13
60	0.0050+/–0.0001	12	0.0177+/–0.0004	41	0.0017+/–0.0003	11	0.0012+/–0.0006	8

measured during the first days of storage. Thus, a microsomal PGHS preparation immobilized on EAE Sephadex retained 40% of initial PGHS activity after one day of storage at 4°C [9]. PGHS immobilized on silica gel without additives retained 34% of activity after 2 days of storage at the same conditions and 29% after 8 days [8]. Similar behavior was observed in the case of PGHS immobilized on pectin film (Table 2). The enzyme retained nearly 40% of activity during one week of storage at 4°C. In the presence of glycerin, the enzyme lost 90% of activity, indicating a destabilization of the enzyme in the presence of this compound. However, in both studied systems, the enzyme retained 11–12% of its initial activity after 60 days of storage at 4°C.

The preparations without glycerin were substantially more stable when stored at lower temperatures (–15°C). The activity of the enzyme in the presence or absence of glycerin was 95 and 88% respectively, on the 5th day of storage. Longer storage led to a dramatic loss in the activity of enzyme preparations with glycerin: after 60 days, the enzyme retained only 8% of its initial activity. Enzyme preparations without glycerin retained up to 41% of activity (Table 2). Thus, PGHS immobilization on pectin films allows for an increase in its stability upon storage up to 60 days.

Study of wound healing kinetics

The kinetics of the progression of wound healing in mice after the application of pectin films with or without immobilized PGHS, as well as healing progression of an untreated wound used as a control are presented in Fig. 1. Pectin film application alone did not affect the kinetics of the repair process. PGHS application significantly improved the progression of wound repair from the first days of treatment and allowed for a decrease in the time needed for wound healing 1.46 fold compared to an untreated wound or a wound treated with pectin film without enzyme (Table 3). The difference in wound healing in a mouse using different treatments (1 week after beginning of the experiment) can be clearly seen

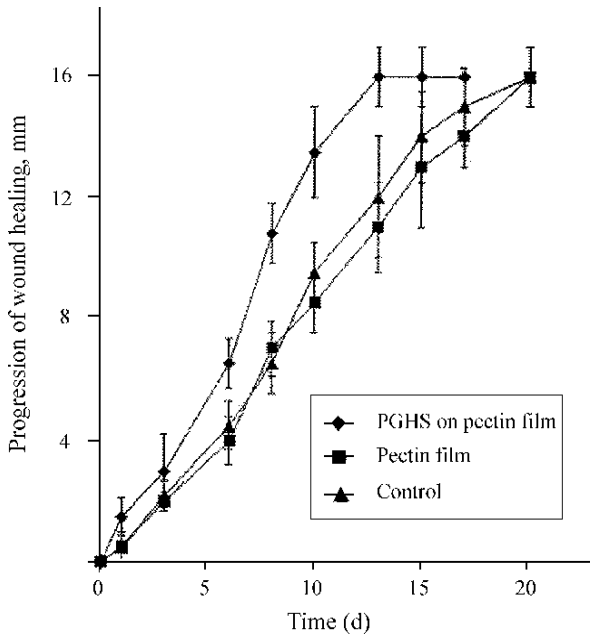

Fig. 1. Kinetics of healing progression for untreated wounds (control) and wounds treated with pectin film with or without immobilized PGHS

Table 3

Time needed for wound healing under different treatments

Type of treatment	Activity of PGHS before application, (O_2)M/min	Time of healing, days
Pectin films with PGHS	8.08×10^{-5}	13+/–3
No treatment	0	19+/–2
Pectin films (PGHS free)	0	19+/– 3

in Fig. 2: the top wound on the back of the mouse was treated with pectin film, the middle wound was the control without treatment, and the bottom was treated with PGHS immobilized on pectin film.

The kinetics of healing progression of surgical linear wounds is similar to the kinetics of microbial or cell culture proliferation: lag-phase, followed by exponential and stationary phases [13]. The sigmoid behavior of curves describing the wound repair process was reported by the Lelcuk S. et al. [3] in an assay carried out with rabbits and extraction of one square of skin.

Fig. 2. Mouse skin wounds at day 7 of the experiment: top wound was treated with pectin film; the middle wound was a control without treatment, and the bottom was treated with PGHS

The linearity of the initial part of the kinetic curves verifies the presence of an exponential function (Fig. 3). As the analogy with the kinetics of cell population growth, the tangent of the resulting lines (μ*) may be interpreted as the specific rate of the process – in this case we used the term "specific rate of healing progression" (μ*).

It was observed that the application of the enzyme increased the specific rate of healing progression (μ*) corresponding to the exponential phase of the kinetics of wound repair that is calculated as the tangent of the inclination angle of lines obtained in semi-logarithmic coordinates (Fig. 3).

A similar assay was performed to study the effect of immobilized PGHS activity on the specific rate of healing progression of linear wounds. For that experiment, PGHS enzyme with different activites were immobilized on pectin films and applied to wounds, so that each wound was treated with PGHS at a different activity. A direct correlation between immobilized enzyme activity and the time needed for wound healing was found . The results are presented in Fig. 4. The linearity in semi-logarithmic coordinates allows the use of tangents to interpret the specific rate of healing progression (μ*) of wounds (Table 4). The dependence of this parameter (μ*) on the applied PGHS activity can be approximated with a hyperbolic function (Fig. 5). The difference of μ* from the control was statistically significant

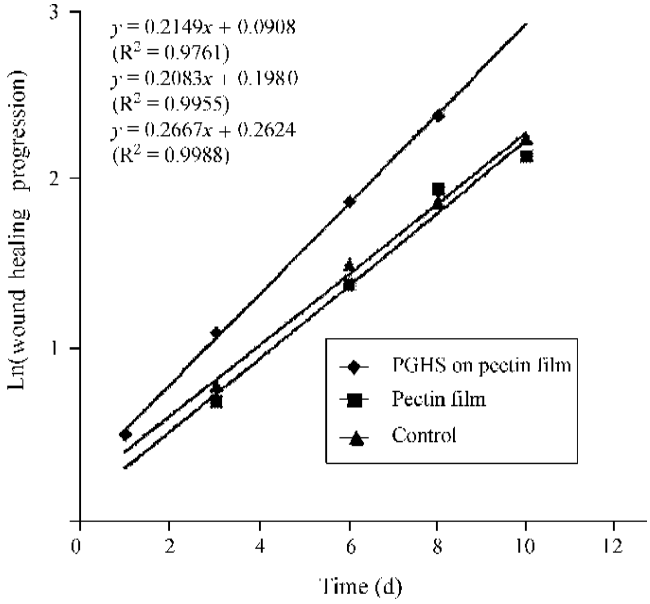

Fig. 3. Linearity of the initial part of the kinetic curves presented in Fig. 1 in semi-logarithmic coordinates

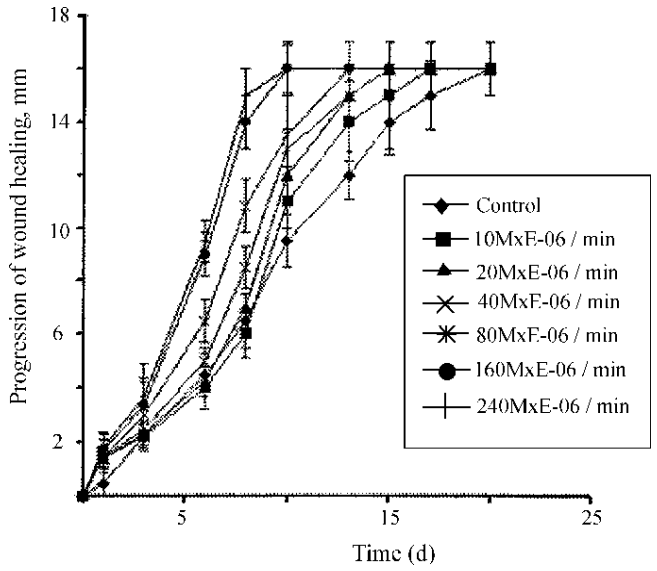

Fig. 4. Kinetics of healing progression determined for untreated wounds (control) and for wounds treated with PGHS of different activities immobilized on pectin films

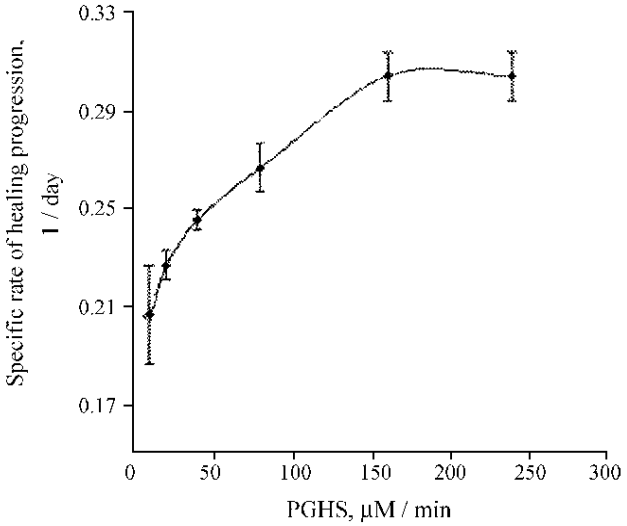

Fig. 5. Specific rate of wound healing calculated as the tangent of lines plotted in semi-logarithmic coordinates as the function of applied PGHS immobilized on pectin film at different activities

at 40 mM/min PGHS activity and higher, while the difference between healing time in the control (untreated) and PGHS treated wounds was statistically significant from 20 mM/min PGHS activity (Table 4).

These results demonstrate that PGHS application stimulates cell growth in the wound related to the prostaglandin synthesis from endogenous substrates.

HPLC measurement of PGE_2 in the wounds

Table 5 shows that application of PGHS significantly increases the level of prostaglandin concentration in treated wounds. In our experiment, we used HPLC to demonstrate that after PGHS application to the wounds for 1 hour, the level of PGE_2 increased 12.97-fold compared to the control wounds without enzyme treatment. So, the effect of PGHS acceleration of wound repair may be related to the regulation of PGE_2 concentration that, according to the literature data, participates as a regulator of cell growth in the skin wound.

Joyce et al. [14] reported that PGE_2 induced angiogenesis in corneal endothelial cells, and they suggested that PGE_2 was a mediator of wound repair. PGE_2, furthermore, has cytoprotective properties against toxic chemicals in various types of tissues [15, 16]. These reports suggest that PGE_2 has the ability to control cytoprotection and the repair of tissues. Skai Y. et al [2] postulated that PGE_2 stimulates angiogenesis and wound healing through the induction of fibroblast growth factor (bFGF). They evaluated the effect of PGE_2 on soft tissue repair by detecting the expression of the mRNA of bFGF, which is one of the well-known factors of angiogenesis and tissue repair. Their findings may provide insight into the mechanism of PGE_2-induced angiogenesis and wound healing in soft tissue through the production of bFGF, in which a receptor for PGE_2 (EP1 or EP2 and EP4) might play an important role [2].

The importance of PGE_2 in the process of wound healing is evident; however, the mechanism of tissue repair or angiogenesis by PGE_2 is not completely understood.

Kinetic model

Various reports [17–19] proposing different mathematical models describing certain aspects of the complex process of wound healing have been published. For example, Maggelakis S.A. described a mathematical model which is based on diffusion equations to describe the dependence of tissue regeneration on oxygen availability, production of macrophage derived growth factors, and the growth of capillary density [20]. Olsen L. et al. investigated a deterministic mathematical model in order to obtain insight into the mechanistic relationships between wound contractions and associated normal and pathological healing processes [21].

In this study, the simplest situation of a wound occurring on the skin was exanimated. The wound was created by a cut and the enzyme responsible for the synthesis of physiological active substances was applied. The acceleration of wound repair was described as a function of the enzyme activity.

It is known that the epidermal wound healing stages include cell migration, cell mitosis, and inhibition of

Table 4

Time and calculated specific rate of wound healing in groups of mice under different treatments

PGHS activity, O_2μM/min	Specific rate (μ*) of wound healing progression, 1/day	Statistic significance (+) – sufficient (–) – insufficient for specific rate	Time needed for healing, days	Statistic significance (+) – sufficient (–) – insufficient for healing time
0 (Control)	0.208±0.01	(data to comparison)	19±2	(data to comparison)
10	0.207±0.02	–	18±1	–
20	0.227±0.006	–	15±1	+
40	0.245±0.004	+	15±1	+
80	0.267±0.01	+	13±2	+
160	0.305±0.01	+	10±3	+
240	0.305±0.01	+	10±2	+

mitosis [21]. uring the cell migration stage, epidermal cells move across the wound area attempting to re-establish the continuity of the epidermis. This stage is followed by a burst of mitotic activity, which provides additional cell population and increases epidermal thickness. Inhibition of mitosis takes place after the appropriate epidermal thickness has been reached [21].

The kinetic sigmoid curves obtained to describe the kinetics of healing progression (Figs 1 and 4) have the exponential phase, similar to the kinetics of cell culture proliferation (Fig. 3). Considering this analogy, we assumed that the exponential phase of healing progression kinetics is related to the mitotic activity process of cell in the damaged skin tissue.

Based on this hypothesis, it was suggested that the proliferation of the cells (*M*) in the tissue might be influenced by the presence of the some compound (*A*). For example, prostaglandins may influence the state of the cell by means of interaction with specific receptors [17]. This type of regulation is common for different hormones, and is related to the formation of the complex between a compound and cell receptor. It is a reversible reaction described by the dissociation constant K_A. Consider that compound *A* may be added to the system by means of an endogenous pathway or may be a product of the enzymatic reaction, for example, a PGHS reaction.

According to Varfolomeev S.. and Kaliuzhniy S.V. [13], the proliferation under saturation of the system with the substrate, may be described by the next scheme:

$$M \xrightarrow{\mu} 2M$$

We propose that the scheme may be applied to describe the cell proliferation in the wound, in case of absence of the regulator compound A. Kinetics is defined by the exponential equation [13]:

$$M = M_0 \exp(\mu t),$$

where parameter μ is a specific rate of the exponential phase of proliferation. Considering that the interaction with the compound *A* takes place, and the complex *AM* characterized by the specific rate of growth (αμ) is different to *M*, it may be written:

$$\begin{array}{l} M \xrightarrow{\mu} 2M \\ + \\ A \qquad\qquad\qquad\qquad\qquad \textit{Scheme I} \\ K_A \updownarrow \\ AM \xrightarrow{\alpha\mu} 2M + A' \end{array}$$

Equation system for *M* concentration is:

$$dM/dt = \mu M$$
$$dM'/dt = \alpha\mu AM$$
$$K_A = A^*M/AM$$

The result of cell proliferation is the progression of scarring or an increase in the total number of cells (*N*):

$$N(t) = M(t) + M'(t). \quad (2)$$

So, the expression for velocity of proliferation using both differential equations of system 1 is:

$$\frac{dN(t)}{dt} = \frac{dM(t)}{dt} + \frac{dM'(t)}{dt}. \quad (3)$$

As well as

$$dN/dt = \mu M + \alpha\mu AM. \quad (4)$$

Using the equation for the dissociation constant (K_A):

$$AM = (A^*M)/K_A. \quad (5)$$

After substitution in equation 2, it was obtained:

$$N(t) = M(t) + \frac{A^* M(t)}{K_A} = M(t)\left(1 + \frac{A}{K_A}\right). \quad (6)$$

Substituting equations 5 and 6 into equation 4 led to the following equation:

$$\frac{dM(t)}{dt}\left(1 + \frac{A}{K_A}\right) = \mu M(t) + \frac{\alpha\mu M(t) A}{K_A}. \quad (7)$$

After separation of variables and integration under condition that at the initial moment $t_0 = 0$, the cell concentration is equal to M_0, equation (8) was obtained:

$$M(t) = M_0 \exp\left[\frac{\mu\left(1 + \frac{\alpha A}{K_A}\right)}{1 + \frac{A}{K_A}} t\right]. \quad (8)$$

The function that describes the kinetics of cell proliferation in the exponential phase may be defined from equations (6) and (9):

$$N(t) = M(t)\left(1 + \frac{A}{K_A}\right) = M_0\left(1 + \frac{A}{K_A}\right)\exp\frac{\mu\left(1 + \frac{\alpha A}{K_A}\right)}{1 + \frac{A}{K_A}}, \quad (9)$$

where at the initial time $t = 0$, the total number of cells is equal to:

$$N_0 = M_0 (1 + A/K_A).$$

Based on the analogy between the equation 9 and Monad's equation, we consider the factor of the potential of the exponential function as the specific rate of proliferation (μ*):

$$\mu^* = \frac{\mu\left(1 + \frac{\alpha A}{K_A}\right)}{1 + \frac{A}{K_A}}. \quad (10)$$

For the case when the compound A interacts with cell receptor but does not affects the proliferation: $\alpha = 1$ and $\mu^* = \mu$, so the specific rate does not change, followed the equation (10).

If the compound A provokes decrease in the proliferation: $\alpha < 1$; followed by the model and the equation (10): $\mu^* < \mu$. So, the specific rate (μ^*) decreases. If A accelerates the proliferation: α 1, and it corresponds to μ^* μ, then the specific rate (μ^*) is increases. In the case of the absence of compound A in the system: $\mu^* = \mu$, the specific rate is unchanged. Moreover, the behavior of μ^* is described by the hyperbolic function of A concentration. Assuming that the compound A is the product of enzymatic reaction, for example, PGHS is responsible for the synthesis of physiological active substance PGE_2, the Michaelis–Menten equation was introduced to the Scheme I.

$$E + S \xleftarrow{K_m} ES \xrightarrow{k_{cat}} A + E \qquad \textit{Scheme II}$$

The scheme II may be analyzed under two different conditions, when ratio between the enzyme and substrate concentrations in the catalytic system: a) E_0 S_0 and b) $E_0 << S_0$.

a) E_0 S_0, in this system the enzyme concentration is much higher than the substrate concentration. In reports about the synthesis of PGs *in vivo*, the enzyme concentration is higher, by various orders, then the level of PUFAs controlled by phospholipase activity [22]. These conditions may be used to describe skin repair process.

Under condition E_0 S_0, the rate of enzymatic reaction as the function of concentration of enzyme and substrate is defined by the equation:

$$V_0 = \frac{k_{cat} E_0 S_0}{K_m + E_0}. \quad (11)$$

So, for the velocity of the enzymatic reaction it may be obtained:

$$\frac{dA}{dt} = -\frac{dS}{dt} = \left(\frac{k_{cat} E_0}{E_0 + K_m}\right) S_0. \quad (12)$$

The differential equation is:

$$\frac{dS}{S} = -\frac{k_{cat} E_0}{E_0 + K_m} dt . \quad (13)$$

The integration of equation (13) leads to the function:

$$S(t) = S_0 \exp\left(-\frac{k_{cat} E_0}{E_0 + K_m} t \right). \quad (14)$$

Considering that $A(t) = S_0 - S(t)$, the equation to define the behavior of A as the time function is obtained:

$$A(t) = S_0 \left(1 - \exp\left(-\frac{k_{cat} E_0}{E_0 + K_m} t \right) \right). \quad (15)$$

Substituting the (15) for equation (10), it is obtained:

$$\mu^* = \frac{\mu \left(1 + \frac{\alpha S_0}{K_A} \left(1 - \exp\left(\frac{-k_{cat} E_0 t}{E_0 + K_m} \right) \right) \right)}{1 + \frac{S_0 \left(1 - \exp\left(-\frac{k_{cat} E_0 t}{E_0 + K_m} \right) \right)}{K_A}}. \quad (16)$$

Equation (16) demonstrates that the behavior of the specific rate μ* is the hyperbolic function of enzyme concentration. If the enzyme concentration is higher than K_m, then the value of μ* does not depend on it. Fig. 6 presents the curve obtained by the computer simulation using the parameters obtained from literature data [13]. Comparison of the experimental results on Fig. 5 and Fig. 6 demonstrates the similar behavior. However, in the presence of skin, the enzyme maintains its activity for 1–2 hours [23] while the wound repair occurs during a period of 13–19 days. It may be important, that the effect of prostaglandins (or other metabolites actuated as activators of cell proliferation) is characterized by the impulsive regulation of the mitotic activity of the cells.

Equation (16) defines that in case of enzymatic synthesis of growth regulator the specific rate of proliferation also depends hyperbolically on the substrate concentration S_0. This observation allows distinguishing between equations (10) and (16), that reflect the effect of direct introduction of the regulator A (equation (10)) and the effect mediated by the synthesis of the physiologically active compound (regulator A), such as PGE_2 or its derivative. The increase of PGE_2 concentration in wounds treated with PGHS was demonstrated (Table 5).

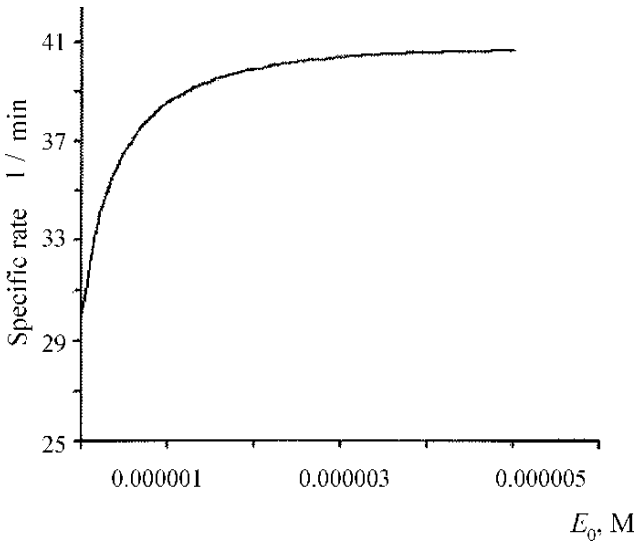

Fig. 6. Simulation of the behavior of specific rate (*) as the function of enzyme concentration responsible of the synthesis of proliferation activator A. Parameters used in equation (16) were: = 30 min^{-1}; K_A = 32 nM; = 1.5; S_0 = 0.5 mM; K_m = 0.32 mM; k_{cat} = 4 sec^{-1}

To distinguish between both models, the assay utilizing arachidonic acid was carried out.

b) The scheme II may be analyzed under the condition that the substrate concentration is much higher than enzyme concentration.

$$S_0 \qquad E_0.$$

In this case, the reaction velocity is defined by the Michaelis–Menten equation:

$$V = \frac{k_{cat} E_0 S_0}{K_m + S_0}. \quad (17)$$

The integration of equation (17) is possible under two approximations. The first case corresponds to situation when: $S_0 << K_m$. Equation (17) may be simplified as:

$$-\frac{dS}{dt} = \frac{k_{cat} E_0 S}{K_m}. \quad (18)$$

After the integration of this differential equation using the initial conditions $t = 0$ and $S = S_0$, it is obtained:

$$S = S_0 \left(\exp\left(-\frac{k_{cat} E_0}{K_m} t \right) \right). \quad (19)$$

Table 5

PGE_2 concentration in wounds treated with PGHS and in control wounds without enzyme treatment

Sample	Weight of skin sample, g	PGHS activity, O_2μM/min	PGE_2, M (calculated from patron plot)	PGE_2 mol/g of skin
Sample treated with PGHS	1.315	373.4	4.44E–07+/–3.51E–07	3.03E–09 +/– 2.40E–09
Control	1.250	0	3.82E–08+/–8.03E–09	2.34E–10 +/– 5.78E–11

This means that the concentration of the enzymatic reaction product A is defined by the equation:

$$A = S_0 \left(1 - \exp\frac{-k_{\text{cat}}\ E_0}{K_m} t\right). \qquad (20)$$

Substitution of this function in equation (10) gives:

$$\mu^* = \frac{\left(1 + \frac{\alpha S_0}{K_A}\left(1 - \exp\left(\frac{-k_{\text{cat}} E_0 t}{K_m}\right)\right)\right)}{1 + \frac{S_0\left(1 - \exp\left(\frac{k_{\text{cat}} E_0 t}{K_m}\right)\right)}{K_A}}. \qquad (21)$$

The behavior of this function is similar to equation (16). It is possible to obtain equation (21) from equation (16) considering that the enzyme concentration is significantly less than K_m ($E_0 << K_m$). Here again the specific rate is dependent of enzyme concentration as well as substrate concentration.

The other case corresponds to the situation when:

$$S_0 \quad K_m.$$

Simplifying equation (17) it may be obtained:

$$-dS/dt = k_{\text{cat}} E_0.$$

Therefore, after integration we have:

$$S = S_0 - k_{\text{cat}} E_0 t. \qquad (23)$$

The concentration of the enzymatic reaction product A is given by the equation:

$$A = S_0 - S_0 + k_{\text{cat}}\ E_0\ t\ = k_{\text{cat}}\ E_0\ t. \qquad (24)$$

Substituting this function in equation (10), it may be obtained:

$$\mu^* = \frac{\mu\left(1 + \frac{\alpha k_{\text{cat}}\ E_0\ t}{K_A}\right)}{1 + \frac{\alpha k_{\text{cat}}\ E_0\ t}{K_A}}. \qquad (25)$$

Equation (25) demonstrates that the specific rate is not dependent on substrate concentration.

The kinetic analysis distinguishes between the scheme I (directed influence) and scheme II, which includes the enzymatic reaction. In addition, the effect of substrate concentration on kinetics of wound repair defines the difference for the conditions: $K_m << S_0 \quad E_0$ (high substrate concentration) and $E_0 \quad S_0$ or $K_m \quad S_0 \quad E_0$ (low substrate concentration). However, the obtained equations do not to distinguish between conditions $E_0 \quad S_0$ and $K_m \quad S_0 \quad E_0$ because in both cases the effect depends on substrate concentration.

Study of effect of arachidonic acid on the kinetics of wound healing

To determine if the effect of PGHS application on wound (Fig. 4) is related to the PGE_2 synthesis (Table 5) and to demonstrate the applicability of kinetic model described previously, the effect of arachidonic acid on wound repair process was studied (Fig. 7). According to the proposed kinetic model (Scheme II), the effect of application of the enzyme must be related to the presence of substrate of the enzymatic reaction and acceleration of cellular proliferation should be modulated (according to proposed mechanism) by arachidonic acid (*AA*) as the precursor of PGE_2. The obtained results (Fig. 7) demonstrate that application of *AA* increases the specific rate of wound healing progression and correlates with proposed model.

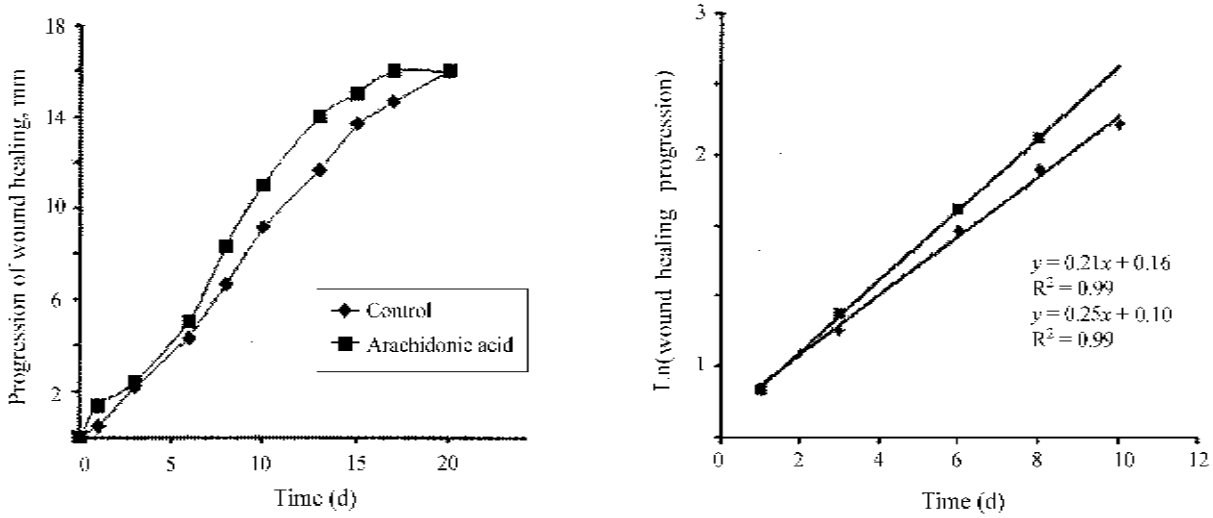

Fig. 7. Linearization of the kinetic curves of healing progression for wounds treated with arachidonic acid at 7 mM and without treatment (control) in semi-logarithmic coordinates

The obtained results demonstrate that the regulation of the level of the prostaglandins is a mechanism to influence on the process of skin wound repair.

The application of PGHS immobilized on pectin film is one of the methods to increase prostaglandin concentration in a wound. Immobilization of PGH-synthase on pectin films allows for stabilization of the enzyme and helps to simplify the procedure of its application on skin lesions. Another method for regulation of prostaglandin concentration is arachidonic acid addition. Based on the obtained results we concluded that the kinetics of the healing process of a linear surgical wound may be described by the typical curve of cellular growth in the closed systems (logistic curve), that probably reflected the dynamics of proliferation of certain groups of cells during the wound healing. The introduction of the parameter of specific rate of wound healing progression allows a quantitative study of wound healing process in the exponential phase, and introduction of the kinetic models describing the effect of PGHS and PGE_2 application on wound repair process.

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IMPROVEMENT OF PGH-SYNTHASE STABILITY IN PECTIN FILMS USED IN HEALING OF SKIN WOUNDS

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Prostaglandin H synthase (PGHS, E.C. 1.14.99.1) and prostaglandins mediate a variety of physiological and pathological processes. Herein, we report a new method of PGHS immobilization applicable to the treatment of skin injuries, using natural polymer pectin as a support matrix. PGHS was isolated from bovine vesicular glands, immobilized on pectin, and tested for stability and activity. The enzyme retained up to 88% of its activity at –15°C during first 5 days and 41% after 60 days of storage. The effectiveness of immobilized PGHS on tissue repair was tested in CD1 mice by measuring linear size of PGHS treated and untreated wound. Application of PGHS accelerated wound healing and stimulated prostaglandin E_2 (PGE_2) generation in the wound. Immobilized PGHS also increased specific rate of healing progression (m*) that was calculated as the tangent of the inclination angle of lines obtained in semi-logarithmic coordinates. A kinetic scheme was proposed to describe the process of wound healing as the function of PGHS concentration.