PharmacologyOnLine
Archives • 2021 • vol.2 • 869-876

EFFECTIVENESS OF VARIOUS PHARMACOLOGICAL DRUGS USED FOR THE TREATMENT OF TRAUMATIC STOMATITIS AGAINST THE BACKGROUND OF DELAYED - ONSET HYPERRESPONSIVENESS – EXPERIMENTAL STUDY

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Abstract

The article presents the results of experimental studies of the effectiveness of prednisolone (PR), cycloferon and solcoseryl under modeling traumatic stomatitis against the background of the development of delayed – onset toxic – allergic hyperresponsiveness (DOHR). It was shown that the combined use of PR and solcoseryl is the most effective in terms of the wound surface healing, smear-imprint from the buccal mucosa, as well as cytological blood markers characterizing the development of allergic reactions.

Keywords: *exudative erythema multiforme, oral mucosa, delayed - onset hyperresponsiveness*

Introduction

Exudative erythema multiforme (EEM) is a disease of the skin and mucous membranes of infectious-allergic and toxic-allergic nature [1]. The reasons for EEM development can be infections (microplasma, herpes, staphylococcus and streptococcus), chemicals, drugs, problems with immunity, chronic diseases of the oral cavity [2, 3]. Until now, there is no single point of view on the etiology and pathogenesis of this disease development.

A number of authors consider it to be a polyetiological pathology, but most authors conclude that allergic genesis plays a leading role in EEM's pathogenesis [4, 5]. Clinically, EEM is manifested by the presence of lesions in the form of round erythematous rings with an external erythematous border and a central blister, between which there is a zone with a normal skin tone [6].

Despite the intensive search for effective methods of EEM treating, there is no single concept in its systemic therapy, and the data published are fragmentary. Therefore, to study the mechanisms of EEM development, creation of adequate experimental models, as well as new approaches to the treatment and prevention of EEM of oral mucosa (OM) remains still relevant in modern dental practice.

Objective: to study the mechanisms and evaluate the effectiveness of prednisolone (PR), solcoseryl and cycloferon use under modeling traumatic stomatitis against the background of the development of delayed – onset toxic – allergic hyperresponsiveness (DOHR).

Methods

The experiments were carried out on white outbred male rats weighing 165-200 g in compliance with all international bioethical requirements [7].

Experimental studies were conducted in accordance with the rules established by the Directive of the European Parliament and the Council (2010/63 / EU), by the order of the Ministry of Education and Science, Youth and Sports of Ukraine No. 249 of March 1, 2012 "On Approval of the Procedure for conducting scientific experiments, experiments on animals by scientific institutions " and methodical recommendation.

The modeling of DOHR of toxic-allergic genesis was carried out by subcutaneous (s/c) administration of dibutyl phthalate (DBP) to experimental animals at a dose of 5.0 mg / kg (1/100 of DL_{50}) in complete Freund's adjuvant (CFA) with its preliminary activation. Provocation was carried out with ½ dose of DBP in CAF on the 24th day of the experiment. The development of allergic process was assessed on the 33rd day and at the end of the experiment by the value of specific agglomeration of leukocytes (RSAL) reaction, general blood analysis and the ratio of the main indicators of the leukogram.

Traumatic stomatitis was reproduced on the 33rd day of the experiment by applying a dosed injury (wound) to the buccal mucosa. Treatment with various drugs was started the next day after the manipulation performed according to the schemes developed. The assessment of the wound's surface healing was carried out after 5 days. A smear-imprint was made and the absolute number of leukocytes and epithelial cells was counted in it.

For the experiment, the animals were divided into 5 groups, 8 animals in each: group 1 – control; group 2 - DOHR model (DBP + CFA) + oral mucosa injury (OMI), 3rd - DOHR model (DBP + CFA) + OMI + intragastric (i / g) PR according to the scheme (36 days); 4th - DOHR model (DBP + CFA) + OMI + intramuscular (i / m) cycloferon according to the scheme (18 days); 5th - i / g PR and intraperitoneal (i / p) solcoseryl according to the scheme (10 days).

Statistical processing of the results obtained was carried out by variation analysis [8] on IBM PC using the software package (Primer of Biostatistics Version 4.03 by Stanton A. Glantz) for Windows and using the Student's t-test to assess statistically significant differences in the groups.

Results

An assessment of allergic reaction development in the dynamics of the experiment is presented in Table 1.

According to the results obtained, administration of DBP in CAF on the 33rd day of the experiment caused the development of an allergic reaction in all experimental groups - RSAL index was 1.93-2.07 points ($p<0.01$). In the control group this indicator was in the range of 1.27-1.33 points.

By the end of the experiment in the 2nd group to which no treatment regimens were applied, the RSAL index tended to further increase in comparison with the 33rd day, and significantly exceeded the values of the control group by 1.6 times ($p<0.001$). After applying the appropriate treatment regimens (for groups 3 - 5), RSAL indicator at the end of the experiment did not significantly differ from the control indicator only in the 4th and 5th groups ($p> 0.5$, $t<2.25$). In the 3rd group there was its significant decrease by 1.2 times ($p<0.05$) compared to the 33rd day, however, in relation to the control, RSAL remained significantly increased ($p<0.05$).

Against the background of the developed allergic reaction on the 33rd day of the experiment, traumatic stomatitis was simulated in animals (the development of a local inflammatory process was initiated) and drugs injections were started in accordance with the developed treatment schemes. Evaluation of the wound surface and the state of local immunity of the OMI was carried out after 5 days. The results of the study are presented in Table 2.

Visual assessment of the wound surface of the buccal mucosa on the 5th day of healing showed that the animals of the 2nd group mainly had incomplete restoration of the cheek epithelium and the assessment of the wound surface averaged 2 points (incomplete restoration of the epithelial layer of the connective tissue of the mucous membrane). In the rest experimental groups (3 -5th), the most positive results of treatment were observed in the 5th group (in comparison with group 2). The average assessment of the wound surface was 0 - 1 points, and this indicates almost complete restoration of defects in the mucous membrane of the cheek epithelium.

In the groups 3 and 4, the results of visual testing showed that the wound surface was still at the stage between "epithelialization" and the restoration of the epithelium. Visual observations of the injured buccal mucosa were confirmed by the results of assessing the number of epithelial cells when taking a cheek smear, as well as by counting the number of leukocytes and the ratio of their individual populations.

In the control group of animals in the study of intact mucous membrane, the ratio of leukocytes / epithelial cells was 89/11, while the ratio of living leukocytes / dead leukocytes was 91/9, and the ratio of living epitheliocyti / dead epitheliocyti was 5/95 (Table 2).

In the 2nd group, when modeling DOHR and damage oral mucosa without treatment on the 5th day after injury, the number of leukocytes and epithelial cells significantly increased by 1.7 and 2.9 times ($p<0.001$), and their ratio also changed (82 / 18), which indicates a protracted course of reparative processes in damaged tissues of the mucous membrane (when the mucous membrane is injured, the wound healing in rats occurs on the 5th day). At the same time, the ratio "live / dead" for leukocytes and epitheliocyti changed up to 53/47 and 10/90 (91/9 and 5/95 in control), which confirms leukocytes activity increase (increase of dead that have fulfilled their function). Increased desquamation of living epithelial cells is associated with incomplete epithelialization of the damaged buccal mucosa.

In animals that were injected with pharmacological preparations according to the developed treatment regimens against the background of DOHR and traumatic stomatitis modeling, a significant decrease in the number of leukocytes and epithelial cells was noted in all groups. However, the most pronounced positive changes were found in group 5 with the combined administration of PR and solcoseryl. At this case they observed a decrease in leukocytes and epithelial cells by 33.6 and 54.7% compared with group 2 ($p<0.001$), as well as by 25.6 and 49.3% compared with groups 3-4 ($p<0.001$). However, at the same time, no significant changes were revealed in the percentage ratio of living / dead cells in relation to group 2 ($p> 0.5$ and $t<2.15$), which also indicates a certain activity of the reparative processes. Significant changes were noted in the percentage ratio of living / dead leukocytes both in relation to both group 2 and 3 - 4 ($p<0.001$) towards stabilization of these indicators. The results obtained indicate a decrease in the intensity of the inflammatory process at varying degrees of severity.

The state of local immunity of the oral cavity was assessed by the ratio of leukocytes on a smear-print taken from the injured cheek. The results presented

in Table 3 show that the relative number of neutrophils in all experimental groups tended to decrease in relation to the control, while monocytes increased only in groups 2-3 (more than twice, $p<0.01$) against the background of a significant increase of lymphocytes by 32.4-42.6% ($p<0.01$).

Lymphocytes and macrophages, migrating to the surface of the mucous membrane, are able to quickly and subtly respond to the slightest changes in the external environment, causing the rapid activation of protective mechanisms. This confirms that the simulated traumatic stomatitis proceeded against the background of immune inflammation of OM tissues, marker indicators of which are leukocytes absolute number increase by 1.7 times, an increase of lymphocytes and monocytes relative quantity, as well as epitheliocyti increase).

When study the mechanisms of specific immunity which are activated after the antigen enters the body, an important role is played by the study of the cellular composition of the peripheral blood, as well as the assessment of leukocytes individual populations ratio. These ratios can be used as general characteristic of cellular reactions of nonspecific and specific body defense. The study of peripheral blood parameters showed that on the 33rd day of the experiment, no Hb significant changes (140.0 ± 16.0 - 156.3 ± 9.9 g / l) or erythrocytes changes (7.65 ± 0.24 - 7.96 ± 0.18 T / l) took place in none of the groups. The same trend was observed at the end of the experiment.

A significant increase in the number of leukocytes by 1.3-1.4 times ($p<0.001$) was found in groups 2-5 on the 33rd day, which may also be a confirmation of the development of allergic reactions in the animals.

By the end of the experiment, this indicator in group 2 had a tendency to further increase, and was significantly higher than the control by 1.5 times ($p<0.001$). In groups 3-5 there was a significant decrease in the number of leukocytes in relation to the 2nd group by 25-29% ($p<0.01$), and in relation to the 33rd day of the experiment - by 18.2 and 20.3 % ($p<0.05$).

The study of the main indicators of the leukogram, presented in table. 4, made it possible to reveal a significant increase in the number of segmented neutrophils and monocytes on the 33rd day of the experiment in all groups by more than 1.4 and 3.6 times ($p<0.01$) in relation to the control, respectively. At the same time, the number of lymphocytes significantly decreased in all experimental groups by more than 1.2 times ($p<0.01$). The values of other indicators (eosinophils and basophils) fluctuated within the control values ($p> 0.5$, $t<2.15$).

After the end of the experiment (Table 5), the number of neutrophils, monocytes and lymphocytes in the 2nd group significantly differed from the control indicators ($p<0.01$). In the groups of animals that were injected with drugs, the dynamics of stabilization of these indicators was observed, however, it varied. In groups 3 and 5 the number of neutrophils significantly decreased in relation to group 2 ($p<0.05$), and the number of lymphocytes did not differ from the control indicators ($p> 0.5$, $t<2.15$), but it significantly differed (towards stabilization) from the indicators of the 33rd day of the experiment ($p<0.05$).

Monocytes decreased most of all in groups 4 and 5, both in relation to group 2 and in relation to the 33rd day of the experiment - by 2.9 and 4.2 times, versus 1.8 in group 3 ($p < 0.05$). It was noted that in groups 3 and 5 at the end of the experiment, there were no basophils, which is probably due to the reaction to PR administration.

Based on the leukocyte formula, the ratios of separate leukocyte populations were calculated, which reflect the cellular balance of nonspecific and specific cellular immunity indexes. The study of the dynamics of changes in the indices of different types of leukocytes showed (Table 6) that when modeling DOHR on the 33rd day of the experiment in the blood of groups 2-5 animals neutrophil / lymphocyte (N / L) indices significantly increased by more than 1.8 times, the indices of neutrophils / monocytes (N / M) and lymphocytes / monocytes (L / M) decreased by more than 2.8 and 5 times, respectively ($p<0.05$). This indicates the activation of the nonspecific link of immunity, development of the inflammatory process, and an increase the role of the macrophage system in the body's defense, i. e. activation of the effector link of the immune response during the development of an allergic reaction after the introduction of DBP into CAF to animals.

At the end of the experiment after various treatment regimens have been used, the most pronounced positive dynamics of N / M and N / L indicators stabilization was revealed in groups 3 and

5. This indicates the restoration of the balance between the specific and nonspecific links of immunity. After the course of therapeutic measures, the restoration of the L / M ratio was noted in groups 4 and 5, which indicates the restoration of the interaction between the effector and affector links of the immune response.

The results obtained agree with the literature data, which show that the development of various dental diseases, including caries, periodontal diseases, viral stomatitis, ulcerative necrotic processes, etc., are based on certain immunological disorders, which, in turn, are the result of local or systemic damage to immune system.

Conclusions

The introduction of DBP in CAF to laboratory animals causes the development of DOHR, which is characterized by increase of indicator of the reaction of leukocytes specific agglomeration to 1.93-2.07 points, versus 1.15-1.32 in the control group ($p < 0.05$), thrice increase of monocytes against the background of an increase of leukocytes total number ($p<0.05$).

This model is adequate for to study the mechanisms of pathophysiological processes development associated with immunological inflammation.

Reproduction of traumatic stomatitis (local inflammatory process) against the background of developed allergic reaction (hyperactivity of the immune system) and administration of various medications showed that restoration of connective tissue epithelial layer, approaching to complete restoration of buccal mucosa defects was observed only in the group of animals that received prednisolone + solcoseryl.

Acknowledgments

The authors declare that there are no conflicts of interest.

References

1. Borodovitsina S.I., Savelyeva N.A., Mezhevikina G.S., Zhiltsova E.E., Filimonova L.B. (2019). Major diseases of the oral mucosa: atlas. Ryazan: OTSiOP, 2019 .- 316 p.
2. Shaik Mohamed Asif, Shaik Mohamed Shamsudeen, Khalil Ibrahim Assiri, Hussain Mohammed Al Muburak, Sultan Mohammed Kaleem, Abdul Ahad Khan, Mansoor Shariff. (2021). Drug induced oral erythema multiforme. Medicine (Baltimore), Apr 30; 100(17).
3. Sarah M. Volkers, Christian Meisel, Dorothea Terhorst-Molawi, Guido J. Burbach, Dirk Schürmann, Norbert Suttorp, Leif E. Sander. (2021). Clonal expansion of $CD4^+CD8^+$ T cells in an adult patient with *Mycoplasma pneumoniae*-associated Erythema multiforme majus. Allergy Asthma Clin Immunol, 17: 17. Published online 2021 Feb 10.
4. Bulgakova A.I., Khismatullina Z.R., Zatsepina M.V., Kudryavtseva Yu.A. (2020). O klinicheskom techenii mnogoformnoy ekssudativnoy eritemy polosti rta (obzor literatury). Parodontologiya, 25(1), 71–74.
5. Jimenez Cauhe J., Ortega Quijano D., Carretero Barrio I., Suarez Valle A., Saceda Corralo D., Moreno Garcia del Real C., Fernandez Nieto D. (2020). Erythema multiforme like eruption in patients with COVID-19 infection: clinical and histological findings. Clin Exp Dermatol, May 9: 10.1111/ced.14281.
6. Pochtar V. N. (2016). The prevalence and features of the course of exudative erythema multiforme. Innovations in Dentistry, N 4 (14), 50-53 [in Russian].
7. Mc Gonigle P, Ruggeri B. (2014). Animal models of human desease: challenges in enabling translation. Biochem. Pharmacol., 87(1): 162–171.
1. 8. Antomonov MY. (2017). Matematicheskaya obrabotka i analiz mediko-biologicheskikh dannykh. 2-e izd. Kiev: MICz Medinform; 2017. 578 p.

Table 1. Changes in RSAL indices in animals in the dynamics of the experiment

Group N	Average reaction score, M mean ± m	
	RSAL indicators on the 33rd day of the experiment	RSAL indicators after the end of the experiment
1st-control	1.33 ± 0.11	1.27 ± 0.13
2nd – DOHR	1.93 ± 0.12 ** /1	2.01 ± 0.12 *** /1
3rd – DOHR + PR	1.98 ± 0.09 *** /1	1.72 ± 0.11 * /1
4th – DOHR + cycloferon	2.03 ± 0.08 *** /1	1.60 ± 0.12 * /2, *** /33
5th – DOHR +PR +solcoseryl	2.07 ± 0.14 *** /1	1.56 ± 0.10 * /2, * /33

Note: /1- in relation to group N 1; /2- in relation to group N 2
/33- in relation to the 33rd day of the experiment
* / - changes are reliable at p< 0.05 and t≥2.15
** / - changes are reliable at p<0.01 and t≥2.98
*** / - changes are reliable at p<0.001 and t≥4.14

Table 2. Assessment of the state of the buccal mucosa in 5 days after modeling traumatic stomatitis against the background of delayed-onset hyperresponsiveness and use of various treatment schemes

Group	evaluation of buccal mucosa wound surface, score	Leukocytes			Epithelial cells		
		Total amount in 1 µL	Live, %	Dead,%	Total amount in 1 µL	Live, %	Dead,%
1-control	0	479.1 ± 50.2	90.8 ± 6.1	9.2 ± 1.1	59.3 ± 3.0	4.8 ± 0.5	95.3 ± 7.9
2-DOHR	1.8 ± 0.4	793.6 ± 77.4 *** /1	52.9 ± 4.3 *** /1	47.1 ± 3.3 *** /1	169.5 ± 14.5 *** /1	10.5 ± 1.9 *** /1	90.0 ± 6.4
3- DOHR +PR	1.5 ± 0.7	708.6 ± 18.0 *** /1, ** /2	47.4 ± 3.4 *** /1	51.4 ± 4.3 *** /1	150.4 ± 3.9 *** /1, ** /2	9.3 ± 0.6 *** /1	90.8 ± 0.6 ***/1
4-DOHR + cycloferon	1.0 ± 0.7	690.9 ± 23.1 ** /1, ** /2	49.1 ± 4.6 *** /1	46.3 ± 2.9 *** /1	156.5 ± 5.5 *** /1	10.1 ± 1.1 ** /1	89.9 ± 1.1 **/1
5 – DOHR + PR + solcoseryl	0.6 ± 0.5	526.8 ± 31.5 *** /2, *** /3, *** /4	73.4 ± 3.8 *** /1, *** /2, *** /3, *** /4	26.6 ± 3.8 *** /1, *** /2, *** /3, *** /4	76.4 ± 3.1 *** /1, *** /2, *** /3, *** /4	7.3 ± 1.1	92.8 ± 1.1

Note: /1- in relation to group N 1
/2- in relation to group N 2
/3- in relation to group N 3
/4- in relation to group N 4;
/- changes are reliable at p<0.05 and t≥2.15
** / - changes are reliable at p<.01 and t≥2.98
*** / - changes are reliable at p<0.001 and t≥4.14

Table 3. Assessment of the state of the buccal mucosa in 5 days after modeling traumatic stomatitis according to leukogram's indicators changes (cheek print-smear)

Group	Segmented neutrophils, %	Monocytes, %	Lymphocytes, %
1st-control	81.5 ± 6.2	0.88 ± 0.23	17.6 ± 1.1
2nd –DOHR	72.9 ± 4.9	2.0 ± 0.26 **/1	25.1 ± 1.4 **/1
3rd –DOHR+PR	73.9 ± 3.1	2.4 ± 0.40 **/1	23.8 ± 0.9 ***/1
4th –DOHR + cycloferon	75.3 ± 4.1	1.5 ± 0.47	23.3 ± 1.3 ***/1
5th – DOHR +PR+ solcoseryl	79.3 ± 1.3	0.9 ± 0.33	19.9 ± 1.4 **/2

Note: /1- in relation to group N 1
/2- in relation to group N 2
* / - changes are reliable at p<0.05 and t≥2.15
** / - changes are reliable at p<0.01 and t≥2.98
*** / - changes are reliable at p<0.001 and t≥4.14

Table 4. Morphological parameters of the animals peripheral blood on the 33rd day of the experiment

Group	Neutrophils		Eosinophils, %	Monocytes, %	Basophils,%	Lymphocytes, %
	Stab	Segmented				
1st-control	2.75±0.78	21.50±1.81	1.0±0.47	1.50±0.47	0.25±0.41	73.00±1.41
2nd –DOHR	3.00±1.15	33.00±2.39 **/1	1.25±0.41	5.75±0.91 ***/1	0.25±0.41	56.8±2.36 ***/1
3rd-DOHR+PR	3.75±1.12	32.88±2.71 **/1	1.25±0.41	5.75±0.78 ***/1	0.13±0.41	56.50±2.39 ***/1
4th-DOHR+cycloferon	4.50±1.05	29.38±2.43 */1	1.38±0.45	5.38±0.65 ***/1	0.25±0.41	59.25±2.60 ***/1
5th – DOHR+PR+solcoseryl	4.13±0.87	30.38±2.96 */1	1.25±0.41	6.25±1.46 **/1	0.25±0.41	57.88±3.33 ***/1

Note: /1- in relation to group N 1
/2- in relation to group N 2
* / - changes are reliable at p<0.05 and t≥2.15
** / - changes are reliable at p<0.01 and t≥2.98
*** / - changes are reliable at p<0.001 and t≥4.14.

Table 5. Morphological parameters of the experimental animals peripheral blood after the end of the experiment

Group	Neutrophils		Eosinophils,%	Monocytes, %	Basophils,%	Lymphocytes, %
	Stabb	Segmented				
1st-control	2.38±0.45	22.13±0.99	1.13±0.31	1.38±0.45	0.25±0.41	72.75±0.91
2nd –DOHR	5.38±1.48	28.63±2.65 */1	1.50±0.47	5.25±0.62 ***/1	0.38±0.45	58.75±2.47 ***/1
3rd-DOHR+PR	2.88±0.73	20.13±1.44 */2,***/33	1.13±0.31	3.13±0.73 */2, */33	0	72.75±1.74 ***/2, ***/33
4th-DOHR+cycloferon	4.00±0.66	24.75±1.86	1.38±0.45	1.88±0.73 **/2, **/33	0.25±0.41	67.88±2.01 */1, */2, */3
5th – DOHR + PR + solcoseryl	2.50±0.47	21.13±1.72 */2, */33	1.38±0.45	1.50±0.66 ***/2,*/33	0	73.50±1.93 ***/2, **/3

Note: $/_1$- in relation to group N 1

$/_2$- in relation to group N 2

$/_{33}$ – in relation to the indexes of the same group on the 33rd day of the experiment;

*/ - changes are reliable at $p<0.05$ and $t\geq2.15$

** / - changes are reliable at $p<0.01$ and $t\geq2.98$

*** / - changes are reliable at $p<0.001$ and $t\geq4.14$

Table 6. The ratio of leukocytes different groups in the blood during the experiment and their changes

Group	On the 33rd day of the experiment				At the end of the experiment			
	N/L	N/M	L/M	L/E	N/L	N/L	N/M	L/E
1-control	0.33 ±0.03	18.0 ±5.3	55.1 ±18.0	59.3 ±29.7	0.34 ±0.02	20.1 ±6.0	59.2 ±16.7	68.2 ±11.3
2- DOHR	0.64 ±0.07 **/1	6.5 ±1.4 */1	10.2 ±1.7 **/1	50.1 ±12.6	0.58 ±0.07 **/1	6.5 ±1.4 */1	11.1 ±1,2 */1	44.5 ±15.0
3-DOHR + PR	0.63 ±0.09 **/1	6.3 ±1.1 */1	10.1 ±1.6 */1	49.4 ±11.5	0.32 ±0.03 **/2, **/33	8.0 ±2.2	25.1 ±6.9	68.2±11.4
4-DOHR+ cycloferon	0.59 ±0.06 **/1	6.5 ±1,3 */1	11.2 ±1.6 */1	48.3 ±14.1	0.43 ±0.04 */33	19.1 ±8.9	43.5 ±17.3	55.2 ±15.6
5-DOHR +PR+solcoseryl	0.60 ±0.09 */1	5.9 ±1.5 */1	9.9 ±2.6 */1	50.7 ±12.3	0.32 ±0.04 **/2, */33	18.9 ±6.6	58.8 ±18.8 */2, **/33	59.8 ±17.0

Note: $/_1$- in relation to group N 1

$/_2$- in relation to group N 2

$/_{33}$- in relation to the indicators of the same group on the 33rd day of the experiment

$/$- changes are reliable at $p<0.05$ and $t\geq2.15$

$**/$- changes are reliable at $p<0.01$ and $t\geq2.98$

$***/$- changes are reliable at $p<0.001$ and $t\geq4.14$.