Effect of methoxyverapamil, diltiazem, morphine and their combination on the formation of irreversibly sickled cells: an *in vitro* study

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تأثيــر ميثوكســـي فيراباميـل ودلتيازيم والمـورفين وتوليفاتـها في تكويـن الخلايا المنجـلية غير القـابلة للتراجع : دراسة في المختبر

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خلاصـة : تم استقصاء تأثير الميثوكسي فيراباميل والـدلتيازيم (وهما من مضـادات الكالـ…يوم) والمورفين (وله مفعول مضاد للكلسيوم) في تكوين الخلايا المنجلية غيـر القابلة للتراجع . فتبين أن إعطاء الميثوكسي فيراباميل في تركيزه العـلاجي ، وفي تركيز أقوى من ذلك بعشـرة أضعاف ، أدى إلى انخفاض تكوّن الخلايـا المنجلية غير القابلة للتراجع بنسـب يعتد بها إحصائـيا وقدرها 12% و21% على التوالي . وقـد أدى الدلتيازيم إلى تخفيض ملحـوظ في هذه الخلايا بينما لم يؤد المورفين إلـى انخفاض يعتد به . وكان تأثير هـذه المواد مجتمعة مماثلا في مجمله لتأثـيراتها منفردة . إن هذه الأدوية يمكن أن تفيد في تخفيض كثـافة أزمات التحوّل المنجلي وأحداث الانسداد الوعائي . ولذلك يقترح تجربتها على الأحياء المصابين بداء الخلايا المنجلية .

ABSTRACT The effect of methoxyverapamil and diltiazem (calcium antagonists) and morphine (calcium antagonist activity) on the formation of irreversibly sickled cells (ISCs) was investigated. Methoxyverapamil at therapeutic concentration and 10 times that level resulted in a 12% and 21% reduction in the formation of ISCs respectively, which was statistically significant. Diltiazem also produced a significant reduction in ISCs but morphine produced no significant reduction. Combination of these drugs produced a net effect similar to their individual effects. These drugs might be useful in decreasing the intensity of sickling crises and vaso-occlusive events. Thus *in vivo* trials in patients with sickle-cell disease are suggested.

Effet du méthoxyverapamil, du diltiazem et de la morphine et de leur association sur la formation des cellules irréversiblement falciformées: étude in vivo
RESUME L'effet du méthoxyverapamil et du diltiazem (antagonistes du calcium) ainsi que de la morphine (activité d'antagoniste du calcium) sur la formation des cellules irréversiblement falciformées a été examiné. Le méthoxyverapamil à la concentration thérapeutique et à 10 fois ce niveau a provoqué respectivement une réduction de 12 % et de 21% de la formation des cellules irréversiblement falciformées, ce qui était statistiquement significatif. La réduction des cellules irréversiblement falciformées en utilisant le diltiazem était importante, par contre, il n'y a eu aucune réduction significative avec la morphine. L'association de ces médicaments a produit un effet global semblable aux effets que chaque médicament a individuellement. Ces médicaments pourraient être utiles pour réduire l'intensité des crises d'anémie et les accidents vaso-occlusifs. Il est donc suggéré d'effectuer des essais in vivo chez des patients atteints de drépanocytose.

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Introduction

Calcium (Ca^{2+}) could have an important role in the pathophysiology of the sickling process. Among sickle cells, irreversible sickle cells (ISCs), which maintain abnormally deformed shape even after prolonged oxygenation, have a three to seven times higher calcium level compared to normal red blood cells (RBCs) [1,2] and twice that of reversible sickle cells [3,4]. There is now increasing evidence that ISCs are involved in the occurrence of vaso-occlusive and haemolytic crises in sickle-cell disease [5–12].

Many of the characteristics of ISCs such as cell shrinkage, membrane stiffness, and reduced osmotic fragility and deformability have all been found to be related to their high calcium level [13,14].

Table 1 Characteristics of patients with homozygous sickle-cell anaemia

	n^a	mean ± s
Sex	11M/11F	
Age (year)	22	24 ± 10
Haemoglobin (g/dl)	20	7.9± 1.5
PCV (%)	20	27.8± 6.5
RBC (per litre)	11	3.8± 1×10¹²
Endogenous ISCs (%)	21	2.6± 2.1
MCHC (%)	20	29.4± 3.9
Patient condition		
Stable	14	
Crisis	8	
Inpatient	12	
Outpatient	10	
Drug history		
No drug	7	
Folic acids	14	
Paracetamol + ibuprofen	7	

^{a}Number of cases for which particular data were available

Because of the obvious role of Ca^{2+} in the sickling process, calcium antagonist drugs have found their place in investigations trying to reduce the number of ISCs, sickling and its detrimental consequences. The calcium antagonist drugs bepridil, nitrendipine, fendilene, nifedipine and verapamil have all been found to have various beneficial effects in *in vitro* studies. However, these drugs have been used in concentrations much higher than their therapeutic levels and the sample sizes have been small; in one study it was one only.

The aim of the present study was to investigate the effect of calcium antagonist drugs and drugs with calcium antagonist activity which have not, so far been studied. These drugs were tested in pharmacological concentrations and in higher concentrations on blood obtained from patients with sickle-cell anaemia. The sample size was larger than in previous studies.

Subjects and methods

Twenty-two patients with homozygous sickle-cell anaemia were recruited for the study. The type of haemoglobin was determined by cellulose acetate electrophoresis. One patient was excluded from the study due to haemolysis of blood samples. Characteristics of the patients are shown in Table 1.

From each patient, 5 ml of venous blood was collected in heparinized tubes. Experiments were performed immediately after blood collection. Preparation of samples and separation of ISCs were performed according to a method described by Ohnishi [15]. The endogenous ISCs were determined by the same method before cell deoxygenation and subtracted from the newly formed ISCs. The drugs investigated

and the concentrations used were as follows:

- methoxyverapamil hydrochloride 0.46 µmol/l, 4.6 µmol/l
- diltiazem hydrochloride 0.74 µmol/l, 7.4 µmol/l
- morphine hydrochloride 0.25 µmol/l, 2.5 µmol/l
- combination of methoxyverapamil hydrochloride 0.46 µmol/l with diltiazem hydrochloride 0.74 µmol/l
- combination of methoxyverapamil 4.6 µmol/l with morphine hydrochloride 2.5 µmol/l

The lower concentrations of these drugs are within the reported pharmacological concentrations and the higher concentrations are 10 times greater.

Preparation of samples

The heparinized venous blood was centrifuged at 1500 g (3000 rpm) for 5 minutes. Plasma and buffy coat were removed by aspiration. The packed cells were washed with normal saline and centrifuged twice in a suspending medium containing 110 mmol/l sodium chloride, 5 mmol/l potassium chloride, 27 mmol/l sodium bicarbonate, 30 mmol/l glucose, 2.4 mmol/l sodium phosphate, 1 mmol/l magnesium chloride and 2% bovine serum albumin. The pH was adjusted to 7.4. The washed erythrocytes were then suspended in the incubation medium (suspending medium plus 2 mmol/l calcium chloride, 200 IU/ml penicillin G and 0.2 mg/ml streptomycin).

Deoxygenation was done by transferring samples of 2 ml of erythrocyte suspension in incubation medium into 2 ml closed plastic tubes. These were incubated with or without drugs in a water bath at 37 °C with slight shaking for 2 hours. After incubation, cell suspensions were oxygenated by room air for 30 minutes to obtain irreversibly sickled cells [15,16].

Separation of ISCs

A 5 ml density gradient solution which contained 53% (V/V) Percoll (Pharmacia, Sweden), 18% (V/V) meglumine iothalamate (Conray 280, May & Baker, England), 27 mmol/l sodium bicarbonate, 1 mmol/l magnesium chloride, 1 mmol/l glucose and 0.5% (W/V) bovine serum albumin at pH 7.4 was centrifuged at 15 000 rpm at 4 °C for 15 minutes in an angle rotor (HI-Spin 21 refrigerated centrifuge, MSE, England) in a 10 ml plastic centrifuge tube to form the density gradient. A 0.4 ml aliquot of incubated erythrocyte suspension (after oxygenation by room air for 30 minutes) was overlayered on top of the preformed density gradient and the tube was then centrifuged again at 3000 rpm for 20 minutes.

Both upper and lower layers were collected separately by aspiration and then washed and centrifuged twice in normal saline (0.9% sodium chloride solution). The shape of the cells in both layers was studied under phase contrast microscope (Bausch and Lomb, Japan) by removing 20 µl of erythrocyte suspension which was then fixed in 20 µl phosphate buffered saline (20 mmol/l NaH_2PO_4, 130 mmol/l NaCl, pH 7.4) containing 2% glutaraldehyde [16]. The cells in the upper layer (lighter layer) consisted mainly of biconcave cells, while those in the lower layer consisted mainly of ISCs. Erythrocytes in each layer were then haemolysed in 5 ml 1% Triton X-100 and the amount of haemoglobin in each layer was measured using a spectrophotometer at 540 nm (Phillips, England). The ratio of haemoglobin found in the lower (heavier) layer to the total amount of haemoglobin was used as an index of the ratio of ISCs to the total erythrocytes [15].

Table 2 The effect of methoxyverapamil, diltiazem, morphine and their combination on the formation of ISCs *in vitro*

Drug	Concentration	n	ISC formation mean ± s	Percentage reduction from control	Significance
Control			21 14.4	±	8.5
Methoxyverapamil	0.46 µmol/l	21	12.6 ± 7.8	12.5%	*
Methoxyverapamil	4.6 µmol/l	21	11.3 ± 7.1	21.5%	*
Diltiazem	0.74 µmol/l	21	13.0 ± 8.0	9.7%	*
Diltiazem	7.4 µmol/l	11	8.9 ± 7.1	38.2%	–
Morphine	0.25 µmol/l	18	12.9 ± 7.1	10.4%	–
Morphine	2.5 µmol/l	18	13.1 ± 7.6	9.0%	–
Methoxyverapamil + diltiazem	(0.46 µmol/l) (0.74 µmol/l)	21	12.5 ± 8.0	13.0%	*
Methoxyverapamil + morphine	(4.6 µmol/l) (2.5 µmol/l)	13	11.2 ± 8.0	22.2%	–

* Significantly different from control
– Statistical analysis was not performed for these parameters because data were incomplete (n = 11,13,18)
s = standard deviation

Statistical analysis

One-way and two-way analysis of variance (where appropriate) was used to evaluate drug and group effects on a personal computer using *Statgraphic statistical package* 2.1. Statistical significance was determined from 95% confidence interval (CI). Differences were considered significant if the 95% CI range did not include zero. For two sample means, t-test for unpaired data was used to evaluate differences in haemoglobin levels, patient condition and percentage of ISCs. Regression analysis was performed on haemoglobin level and ICS percentage.

Results

The calcium channel blocker, methoxyverapamil, at 0.46 µmol/l caused a 12.5% reduction in the formation of ISCs compared to the control. This reduction was statisti-
cally significant (mean difference from control −1.8 ± 2.15; 95% CI −3.07 to −0.54). The effect of methoxyverapamil seemed to be concentration-dependent. A tenfold increase in the concentration of the drug to 4.6 µmol/l resulted in almost doubling the reduction in the formation of ISCs (21.5%) which was also statistically significant (mean difference from control −3.1 ± 2.84; 95% CI −4.34 to −1.82) (Table 2).

Diltiazem at the lower concentration (0.74 µmol/l) produced a 9.7% reduction in the formation of ISCs compared to the control. This reduction was statistically significant (mean difference from control −1.4 ± 2.34; 95% CI −2.65 to −0.12). The higher concentration of diltiazem (7.4 µmol/l) produced a further reduction in formation of ISCs (Table 2). However, statistical analysis was not done because of the relatively small sample size ($n = 11$).
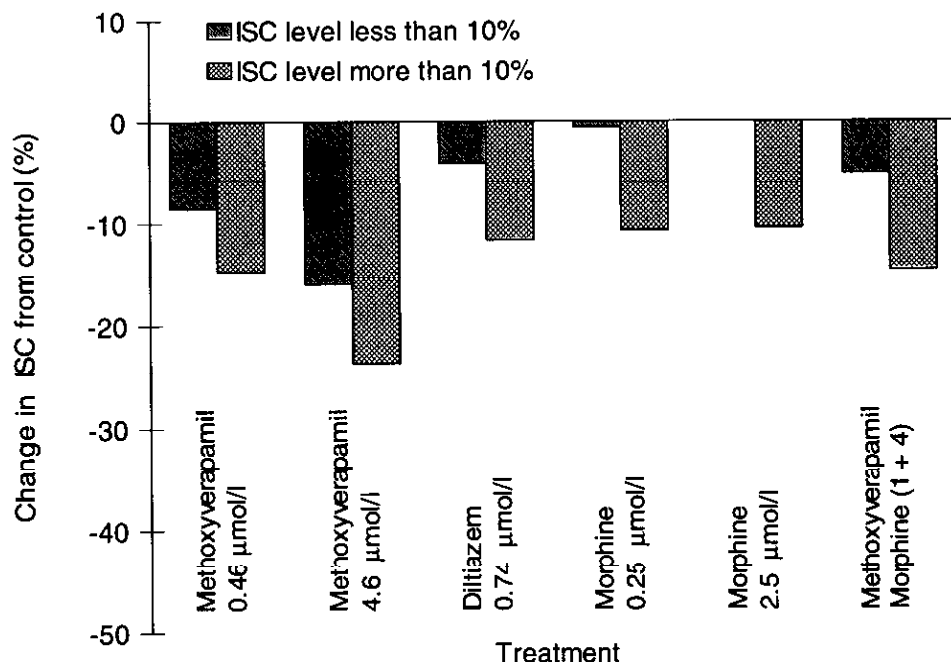
Figure 1 Percentage change from control in ISCs formed during deoxygenation of HbSS cells incubated with drugs. The study population was arbitrarily divided according to the pretreatment ISC levels into <10% and >10%

Morphine at both concentrations (0.25 µmol/l and 2.5 µmol/l) produced a small and statistically insignificant reduction in ISC formation (Table 2).

The effect of combining low concentrations of methoxyverapamil 0.46 µmol/l and diltiazem 0.74 µmol/l was similar to the effect of methoxyverapamil alone and showed about a 13% reduction in ISC formation. This reduction was statistically significant (mean difference from control -1.9 ± 3.88; 95% CI –3.07 to –0.54). The combination of methoxyverapamil (4.6 µmol/l) with morphine (2.5 µmol/l) produced a big reduction in ISC formation. The results are presented in Table 2.

In order to investigate the effect of the pretreatment level of ISC percentage on the response to various drugs, the sample population was arbitrarily subdivided into two groups: those with low pretreatment ISC level (ISCs < 10%) and those with ISCs > 10% [17]. The magnitude of the reduction in the formation of ISCs was greater in the group of patients with ISCs > 10% although it was not statistically significant. For example methoxyverapamil at 4.6 µmol/l resulted in a 23.6% reduction in the formation of ISCs in the group with ISCs > 10% compared to a reduction of 16.1% in the group with ISCs < 10% as shown in Figure 1.

The level of haemoglobin was also investigated. It was found that patients in crisis had significantly lower haemoglobin levels ($P < 0.02$). Regression analysis was
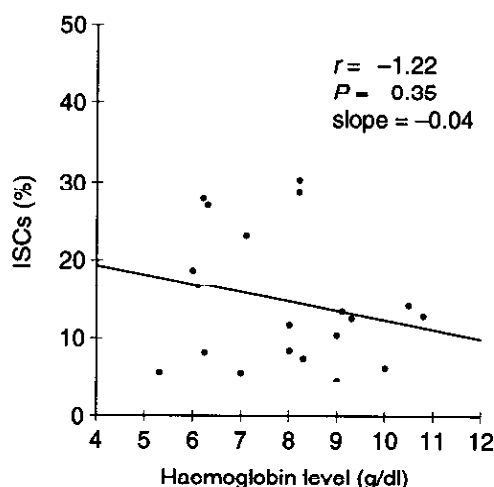
$r = -1.22$
$P = 0.35$
slope $= -0.04$

Figure 2 **Correlation between percentage of ISCs and haemoglobin level**

done between haemoglobin level and percentage of ISCs. There was a negative but statistically insignificant correlation ($r = -0.22$; $P = 0.347$) (Figure 2).

Discussion

Sickle-cell disease is common in the Eastern Mediterranean Region. Prevalence rates of around 13% and 25% have been reported in the southern part of Iraq [18] and among workers in the eastern province of Saudi Arabia respectively [19]. Although specific treatments have, so far, proved disappointing [20], promising results with the anti-cancer drug hydroxyurea have recently been reported [21].

The results obtained from the present study showed that calcium antagonist drugs methoxyverapamil and diltiazem can reduce the formation of ISCs *in vitro*.

It has been reported that ISCs and dense cells are particularly important in the vaso-occlusive state [22] and can induce sickling

with a small number of polymers even at normal oxygen tension [23]. Therefore, the reduction in ISCs found in the present study (12%–21%) although small might be of clinical significance in preventing such vaso-occlusion caused by these cells in patients with sickle-cell disease.

In a small study which was conducted on one blood sample taken from a patient with sickle-cell anaemia, the calcium antagonist drugs nifedipine, nitrendipine and verapamil were found to inhibit the formation of ISCs [17]. In the study, the effect of these drugs was tested using high drug concentrations which for verapamil, for example, was up to 1000 times its therapeutic level. Although, verapamil at 10 times its therapeutic level produced about a 15% reduction in ISCs, complete inhibition of ISCs was achieved at 1000 times that concentration [17]. In our study we used methoxyverapamil at a pharmacological concentration of 0.46 µmol/l and 10 times this concentration, 4.6 µmol/l (pharmacological concentration of methoxyverapamil is 0.2 µmol/l–0.5 µmol/l). The same was applied for diltiazem: 0.74 and 7.4 µmol/l. The combination of methoxyverapamil with diltiazem did not change the effect on ISCs formation and caused a reduction in ISCs similar to that of methoxyverapamil alone.

Morphine was included in the study because it can be used as an analgesic drug to relieve painful crises. In addition, although it is not known whether morphine has an effect on erythrocytes, it has an inhibitory effect on calcium influx in the brain [24]. Overall, morphine caused no significant reduction in ISC formation.

Patients in crisis were found to have low haemoglobin levels. In addition, there was an inverse relationship between haemoglobin level and percentage of ISCs. Thus, low haemoglobin levels in such patients could

be due to a high percentage of ISCs since ISCs are responsible for haemolytic state in (sickle-cell anaemia) patients [5,25]. It is known that ISCs have high calcium levels [1] and the presence of external calcium is important in the formation of ISCs *in vitro* [14,15,17]. This involvement of calcium provides a basis for the activity of calcium channel blockers, although the exact mechanism by which these drugs inhibit the formation of ISCs is not well established. The effect may be due to inhibition of the calcium activated potassium channel (Gardos channel) [26,27]. This channel is activated by increased calcium influx during deoxygenation of sickle-cell erythrocytes which leads to potassium loss and cell dehydration [28,29]. The Gardos phenomenon has been described as one of the most important pathways for the formation of ISCs [14]. Nifedipine and nisoldipine are both calcium channel blockers of the same group. Nifedipine but not nisoldipine has been shown to inhibit ISCs formation [14]. Other agents with calcium channel blocking activity such as lanthanum (La^{3+}) and zinc (Zn^{2+}) have not been found to affect ISCs formation [17]. This lack of activity by some calcium channel blockers may be explained by the finding that calcium influx into the sickle cell occurs not only through calcium channels that are inhibited by calcium channel blockers [30]. Additionally, drugs with no calcium channel blocking activity such as cepharanthine have been shown to inhibit the formation of ISCs [15].

The observation in this study that calcium channel blockers produced a greater reduction in the formation of ISCs in the group with high levels of ISCs (>10%) may indicate that calcium channel blockers are more effective in circumstances where calcium involvement is maximum as in sickle cells. Thus, calcium channel bolckers seem to be more effective in reducing the formation of ISCs in patients in crisis than those in stable condition. The use of calcium antagonist drugs *in vivo* could have an additional important effect through peripheral vasodilatation which might enhance the flow of blood and reduce vaso-occlusive events. However, these drugs need to be tested in patients during acute sickle crisis and in patients with sickle-cell disease.

In conclusion, these drugs can protect erythrocytes from the deleterious consequences of an elevated concentration of intracellular calcium. Therefore, they could be of importance in the management of sickle-cell disease, and may also lend themselves to combination therapy with other promising therapeutic agents such as hydroxyurea.

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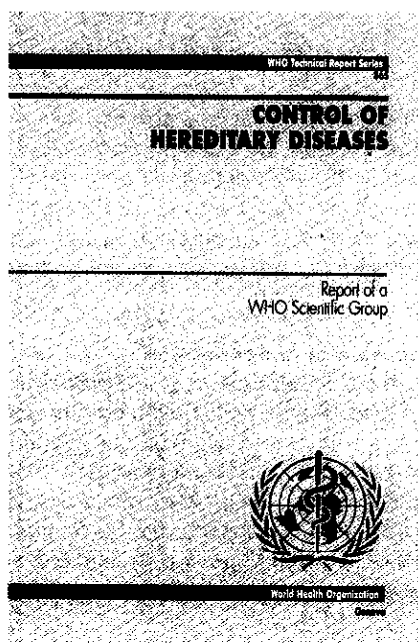
Possibilities for treating – or, ideally, avoiding – hereditary disease have increased enormously with the rapid and radical developments in the field of molecular biology. This report of a WHO Scientific Group deals first with the human genome and the genetic basis of disease, then continues with a review of the epidemiology of genetic disorders and the role of genetic predisposition in various common conditions. In discussing prevention, the report considers both genetic family studies and population screening. It is pointed out that the cost-benefit ratio of providing genetic services is extremely favourable, and prevention is often far cheaper than treatment.

A section is devoted to the important area of genetic counselling. The obstetric aspects of prenatal diagnosis, the organization of genetic services and the ethical, social and legal aspects of genetic technology in medicine are examined. The report concludes with a summary of the Scientific Group's recommendations.