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Gelatin microspheres crosslinked with D,L-glyceraldehyde as a potential drug delivery system: preparation, characterisation, in vitro and in vivo studies

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Abstract

To overcome the restriction in using crosslinked gelatin in the pharmaceutical field, D,L-glyceraldehyde (GAL), a non-toxic crosslinking agent, was proposed. Gelatin microspheres crosslinked with different concentrations of GAL (0.5, 1 or 2%, w/v) and for different time periods (1 or 24 h) were prepared. The effect of the preparation variables was evaluated analysing the extent of crosslinking, the morphological aspect, the particle size and the swelling behaviour. To evaluate the pharmaceutical properties, an antihypertensive drug, clonidine hydrochloride, was chosen as drug model and loaded into the microspheres. Either the increase of the crosslinker concentration or of the crosslinking time period decreased both the swelling and the in vitro drug release processes of the microspheres. After the subcutaneous injection, the loaded microspheres crosslinked with the lowest GAL concentration (0.5%, w/v) or for the shortest time period (1 h) showed a reduction of systolic blood pressure (SBP) similar to that recorded with a clonidine hydrochloride solution having the same drug concentration. Instead, the microspheres crosslinked for 24 h with concentrations of GAL higher than 0.5% (w/v) produced a more gradual and sustained SBP reduction and the antihypertensive effect was maintained until 52–72 h. The biocompatibility studies showed that the microspheres crosslinked with GAL are well tolerated in vivo. These results suggest the potential application of gelatin microspheres crosslinked with GAL as a suitable drug delivery system for the subcutaneous administration.

Keywords: Antihypertensive effect; Biocompatibility; Clonidine hydrochloride physical properties; D,L-Glyceraldehyde; Drug release; Gelatin microspheres; Matrix swelling

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1. Introduction

Gelatin microspheres have been widely investigated for drug controlled release (Tanaka et al., 1963; Leucuta, 1986; Jeyanthi and Panduranga Rao, 1987; Tabata and Ikada, 1989; Forni et al., 1992a,b; Leucuta et al., 1997). The biocompatibility and the degradation to non-toxic and readily excreted products were the main attractive characteristics of gelatin, which suggested its use in the drug delivery field. But, being a soluble polymer, gelatin has to be modified to prepare drug delivery systems. Thus, to obtain a hydrophilic polymer insoluble at 37°C, the chemical formation of crosslinks among the macromolecular chains has been proposed. Either aldehydes, such as formaldehyde (Chemtob et al., 1988; Vandelli et al., 1991), glutaraldehyde (Raymond et al., 1990) or other bifunctional reactives (Robinson, 1964), were used to produce insoluble biodegradable gelatin microspheres. Unfortunately, the safety of these crosslinking agents in humans is not clearly shown and cytotoxic effects were observed for natural polymers crosslinked with aldehydes such as glutaraldehyde (Speer et al., 1980).

In the last years, gelatin crosslinked with D,L-glyceraldehyde was proposed as a new material for pharmaceutical applications (Forni et al., 1992a,b) The use of this aldheyde was suggested to overcome the restriction in using crosslinked proteins due both to the supposed biodegradation to toxic products and to the presence of residuals of crosslinking agent, which is usually toxic. In fact, D,L-glyceraldehyde can be considered a non-toxic crosslinking agent [DL50 i.p. in rat 2000 mg/kg (Görög et al., 1965)]. Moreover, the D-form of this aldehyde is phosphorilated by triosekinase in the human body forming D-glyceraldehyde-3-phosphate that enters in glicolysis cycle.

Till now, both the swellable (Vandelli et al., 1995) and the bioadhesive properties (Vandelli et al., 1996) of the gelatin microspheres crosslinked with D,L-glyceraldehyde have been investigated.

In the present study, gelatin microspheres crosslinked with D,L-glyceraldehyde have been prepared and investigated in order to predict their potential as sustained delivery system for the subcutaneous drug administration. Different concentrations of the crosslinker agent and different time periods of the crosslinking process were used to change the extent of gelatin crosslinking and hence to modify the drug release. As a model, an antihypertensive drug, clonidine hydrochloride, was used. Thus, the effect on blood pressure, obtained after the subcutaneous injection of different microsphere samples in male spontaneously hypertensive rats was recorded and compared with that of the solution of clonidine hydrochloride alone. Biocompatibility is primary concern in designing a subcutaneous drug delivery system. Hence, investigations were undertaken for assessing the immediate short-and long-term response of the tissues after the subcutaneous injection of the gelatin microspheres in female albino guinea pigs.

2. Materials and methods

2.1. Materials

Gelatin (225 Bloom) from calf skin (batch CW 05314MV, Aldrich Chemical Co., Milwaukee, WI) was used without further purification. The mean molecular weight was 176 654 Daltons. The viscosity and the pH of the Bloom solution at 60°C were 4.1 mPa s and 5.6, respectively. D,L-glyceraldehyde (2,3-dihydroxypropionaldehyde, MW 90.08) (GAL) (Aldrich Chemical) and cottonseed oil (Sigma Chemical Co., St. Louis, MO) were used as received from the manufacturers. Clonidine hydrochloride (Lusochimica, Milan, Italy) was the drug. Male spontaneously hypertensive rats (SHR; Charles River, Calco, Italy) and female albino guinea pigs (430 ± 30 g) (Hartley Strain; Morini, S. Polo d'Enza, Italy) were used to evaluate the antihypertensive effect and the microsphere biocompatibility, respectively. All the solvents were of analytical grade (Carlo Erba, Milan, Italy).

2.2. Preparation of the unloaded microspheres

Cottonseed oil (40 ml) preheated to 60°C was added dropwise to an aqueous solution of gelatin

[6 g (3.4×10^{-5} mol)/20 ml] at 60°C, forming an emulsion by stirring with a two-paddle stirrer (about 800 rpm). As the emulsion was obtained, the temperature was lowered to 5°C by rapid cooling in an ice bath. Then, to solidify completely the droplets of the dispersed phase, 60 ml of precooled (5°C) acetone was added, stirring for at least 10 min. Once formed, the microspheres were filtered, washed with cool acetone (5°C) and rapidly dried. To crosslink the gelatin microspheres, different concentrations of GAL (0.5, 1 or 2%, w/v; equivalent to an amount of 1, 2 and 4×10^{-3} mol) were used. Practically, the uncrosslinked microspheres were suspended under magnetic stirring (800 rpm) and for different time periods (1 h or 24 h) in 60 ml of acetone-water (40:20, v/v) solutions containing different amounts of GAL (1, 2 and 4×10^{-3} mol). The temperature was kept at 5°C throughout the procedure. After the crosslinking procedure, the microspheres were filtered, quickly washed with acetone precooled to 5°C and then vacuum-dried (10 mmHg) for at least 24 h. For each sample at least three batches were prepared.

2.3. Preparation of the drug-loaded microspheres

To load clonidine hydrochloride in the crosslinked gelatin microspheres, the swellable properties of the drug carrier were used (Vandelli et al., 1995). Briefly, the drug was loaded allowing the microspheres (1 g) to swell at 37 ± 0.2°C under continuous magnetic stirring (200 rpm) in a water solution of clonidine hydrochloride (0.5 g/100 ml) for 15 min. After this time period, the drug-loaded microspheres were collected by rapid filtration, washed with water and then lyophilised. For each sample at least three batches of loaded microspheres were prepared.

2.4. Residuals of the preparation analysis

Free GAL residue in the microspheres was determined by a gaschromatographic method after the conversion of the aldehyde into the corresponding oxime and the following trimethylsilylation as reported in one of our previous works (Griffini et al., 1996). The acetone residue was also analysed using a gas-chromatographic method after the preliminary treatment of the microspheres in an ultrasonic bath (model 3200; Branson Ultrasonic, Danbury, CT) for 30 min with 1 ml of *N*,*N*-dimethylformamide (Merck, Darmstadt, Germany) containing 1 mg of *n*-propylacetate (BDH Schilling Poole, UK) as the internal standard according to the method previously reported (Griffini et al., 1996).

2.5. Extent of crosslinking evaluation

The determination of free amino groups of gelatin microspheres before and after the treatment with GAL has been used to evaluate the extent of crosslinking. Practically, according to Ofner and Bubnis (1996), an exactly weighed amount of microspheres (11 mg) was treated for 4 h at 40°C with 1 ml of a $NaHCO_3$ (Carlo Erba) (4% w/v) water solution and 1 ml of a trinitrobenzensolfonic acid (TNBS) solution (Fluka Chemie, Buchs, Switzerland). Then, HCl 6N (3 ml) was added and the mixture was autoclaved (model NF 732 FV/2, Fedegari Autoclavi, Albuzzano, Italy) for 1 h at 120°C and 15–17 psi. The hydrolysed product was diluted with water (5 ml) and extracted with ethyl ether (5 ml) and an aliquot of the aqueous phase (5 ml) was first warmed at 37°C for 15 min and then cooled to room temperature and diluted again with 15 ml of water. The moles of the free amino groups/g of gelatin were determined by spectrophotometrical determinations at the wavelength of 346 nm (model Lambda 3B, Perkin–Elmer, Norwalk, CT) against a blank using the relationships proposed in the literature (Ofner and Bubnis, 1996). The reference solution (blank) was prepared as described above, but adding HCl 6N before TNBS in order to block the TBNS reaction with the free amino groups of gelatin. All the data were the average of at least six determinations.

2.6. Morphological analysis

The morphology of the gelatin microspheres was determined by observation of the samples on a scanning electron microscope (SEM) (XL 40, Philips, Eindhoven, The Netherlands). The sam-

ples were mounted on aluminium stubs (TAAB Laboratories Equipment, Berks, UK) using double side sticky tab (TAAB Laboratories Equipment) and vacuum coated with gold-palladium in an argon atmosphere for 60 s (Sputter Coater Emitech K550, 2M Strumenti, Rome, Italy)

2.7. Particle size measurement

Particle size measurements of the gelatin microspheres were performed in triplicate using a mercury porosimeter (model 2000, Carlo Erba) (Carli and Motta, 1984).

2.8. Dynamic swelling

The dynamic swelling process of the microspheres was studied by measuring the amount of water absorbed by an exactly weighed amount of the sample (100 mg) according to the method previously proposed by Nogami et al. (Nogami et al., 1969). Briefly, the dried microspheres were placed on the glass filter of the Enslin apparatus and the volume of water absorbed at 37 ± 0.2°C was observed. The values (mean of at least 4 experiments) were expressed as ml of water absorbed/g of microspheres.

2.9. Drug content

The amount of clonidine hydrochloride loaded in the microspheres was determined spectrophotometrically (Lambda 3A, Perkin-Elmer) at the wavelength of 272 nm after both the drug extraction in deionised water and the microsphere dissolution in HCl 10N. The amount of clonidine hydrochloride in the microspheres was also determined microanalytically on the basis of the percentage of chlorine (White, 1982). The data obtained using the different methods differed by a maximum of $\pm 3\%$.

2.10. In vitro release studies

The dissolution tests were examined using a column-type apparatus (Apparatus 4 USP) (Dissotest CE-1, Sotax, Basel, Switzerland) in 100 ml of deionised water at a flow rate of 25 ml min^{-1}. All experiments were carried out under sink conditions at a temperature of 37 ± 0.2°C using 100 mg of gelatin microspheres. Drug content in the solution was determined spectrophotometrically (Lambda 3A, Perkin–Elmer) at the suitable wavelength (272 nm). All experiments were performed in triplicate and results were in agreement within 3.5% error. The average values are reported in the figures.

2.11. Antihypertensive effect studies

The experiments were carried out on groups of at least six male spontaneously hypertensive rats of about 12 weeks (mean weight 220 ± 25 g; mean blood pressure 200 ± 10 mmHg). A 0.5 ml of either a saline solution (0.9% w/v NaCl) of clonidine hydrochloride (0.04 mg/kg) or a suspension in saline solution of an exactly weighed amount of microspheres containing the same dose/kg of drug were injected subcutaneously in each rat. Systolic blood pressure (SBP) was recorded by a tail cuff method using a BP Recorder (Basile, Comeno, Italy) (Gerold and Tschirky, 1968). SBP was measured immediately before the experiment and 1, 2, 4, 6, 8, 12, 24, 36, 48 and 72 h after the injection. The experimental data were statistically analysed and compared by the paired t test.

2.12. Biocompatibility studies

A suspension (0.5 ml) in saline solution (0.9% w/v NaCl) of unloaded gelatin microspheres crosslinked with GAL 2% w/v for 24 h (4 mg/kg) was injected subcutaneously in the right fore leg of female albino guinea pigs (430 ± 30 g). The microspheres were previously sterilised by immersion in absolute alcohol for 30 s (Jeyanthi and Panduranga Rao, 1990). The animals were maintained on standard diet and water ad libitum. Immediately before the experiment, the guinea pigs were anaesthetised with diethyl ether and prepared by shaving their fore leg and then scrubbing with ethyl alcohol. Then, the control animals received the saline solution alone (1 ml), while the microsphere suspension was injected in the other animals. Four guinea pigs, one of which was the

control, were killed periodically at 24 and 72 h, 1 and 2 weeks, 1 month, 3 and 6 months after the injection of the microsphere sample or of the saline solution. Then, a part of the subcutaneous tissue surrounding the injection site was excised (4 cm in diameter; deep about 0.5 cm). The tissue was firstly fixed in formalin 10% (p/v) and embedded in paraffin wax (Histoplat m.p 56–58°C, Shandon Lipshaw, Pittsburgh, Pa). Then, sections (about 2 mm) were cut using a microtome (model Jang SM 2000R, Leitz, Wetzlar, Germany) (section thickness 0.1 mm) at different levels in the block and stained with eosin/haematoxylin. The inflammatory response was evaluated by optical microscopy (Axiophot, Zeiss, Jena, Germany) fitted with a microphotographic attachment.

3. Results and discussion

3.1. Residuals of the preparation

The results of the gas-chromatography analysis showed that the amount of acetone in the microspheres was generally lower than the detection limit (10 ppm). A residue of 35 ± 7 ppm of free D,L-glyceraldehyde was detected in the different microsphere samples. The detection limit of the free D,L-glyceraldehyde in the microspheres was 16 ppm.

3.2. Extent of crosslinking

The reaction of D,L-glyceraldehyde with proteins involves the reactive groups of a protein, especially the amino groups (Forni et al., 1992a,b). Hence the free amino groups of gelatin microsphere sample uncrosslinked or crosslinked with GAL have been determined.

Table 1 lists the free amino groups of gelatin before and after the treatment of the microphores with GAL. The tabulated values indicate for all the microsphere samples a decrease of free amino groups of gelatin after the treatment with GAL. Varying the GAL concentration from 0.5 to 2% (w/v), a loss of 22–60% of amino groups was evident in the gelatin microspheres treated for a time period of 24 h. Increasing the crosslinking time period from 1 to 24 h, the gelatin microspheres treated with 2% (w/v) of GAL showed a loss of 30–60% of amino groups. As the number of the amino groups lost after the treatment with GAL is representative of the extent of crosslinking, it should be concluded that the crosslinking degree in gelatin microspheres treated with GAL increases, increasing either crosslinker concentration or crosslinking time period.

Table 1
Values of uncrosslinked aminogroups (moles/g) in the different gelatin microsphere samples[a]

Gelatin microspheres	Free amino groups (moles/g $\times 10^5$)
Uncrosslinked	25.2 (0.4)
Crosslinked with 0.5% of GAL for 24 h	19.6 (0.8)
Crosslinked with 1% of GAL for 24 h	17.4 (0.5)
Crosslinked with 2% of GAL for 24 h	10.2 (0.2)
Crosslinked with 2% of GAL for 1 h	18.9 (0.6)

[a] S.E. error in parentheses.

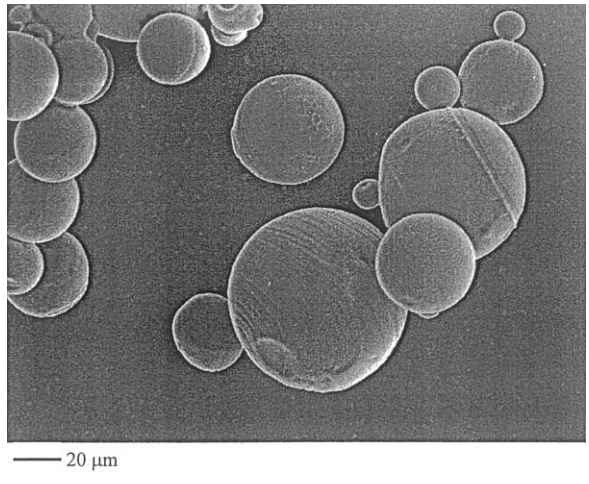

Fig. 1. SEM photomicrograph of gelatin microspheres crosslinked with D,L-glyceraldehyde.

Table 2
Average diameter (μm) of the different crosslinked gelatin microsphere samples[a]

Gelatin microspheres	Microsphere diameter (μm)
Crosslinked with 0.5% of GAL for 24 h	48 (10)
Crosslinked with 1% of GAL for 24 h	43 (9)
Crosslinked with 2% of GAL for 24 h	44 (11)
Crosslinked with 2% of GAL for 1 h	41 (12)

[a] S.E. in parentheses.

3.3. Microsphere morphology and particle size

The SEM photomicrographs of the gelatin microspheres treated with GAL (Fig. 1) showed a very smooth and uniform surface. An average particle diameter of about 40 ± 11 μm resulted from the particle size analysis (Table 2). Both the appearance of the surface and the particle size of the microspheres were not affected by the experimental crosslinking conditions (time, croslinking concentration) or by the drug loading.

3.4. Dynamic swelling

The volume of water absorbed by the gelatin microspheres increased monotonically until reaching the equilibrium swelling value (d_∞) (Fig. 2). As previously reported (Vandelli et al., 1995), both the crosslinker concentration and the crosslinking time period affect the water absorpion of gelatin microspheres (Table 3). The lowering of the volume of water absorbed from the microspheres, increasing the crosslinker concentration or the crosslinking time period, is an evidence of the reduction in the mobility of the macromolecular chains of gelatin. The change in the gelatin crosslinking degree as demonstrated by the analysis of the free amino groups of the gelatin microspheres can explain their water absorption behaviour. Observing the water absorpion profile of the gelatin microspheres at 37°C, a time period of 15 min was chosen for the drug loading process.

3.5. Drug content

The amount of clonidine hydrochloride loaded in the microspheres ranged from 3 to 4 mg/100 mg of microspheres (Table 4). The drug content in the microspheres was only slightly affected by the crosslinker concentration, whereas no difference in the clonidine hydrochloride payload was evident as the crosslinking time period increased. Similar results were previously pointed out for gelatin microspheres crosslinked with formaldheyde and loaded with clonidine hydrochloride (Vandelli et al., 1991).

3.6. In vitro release studies

The release profile of clonidine hydrochloride from gelatin microspheres crosslinked with GAL

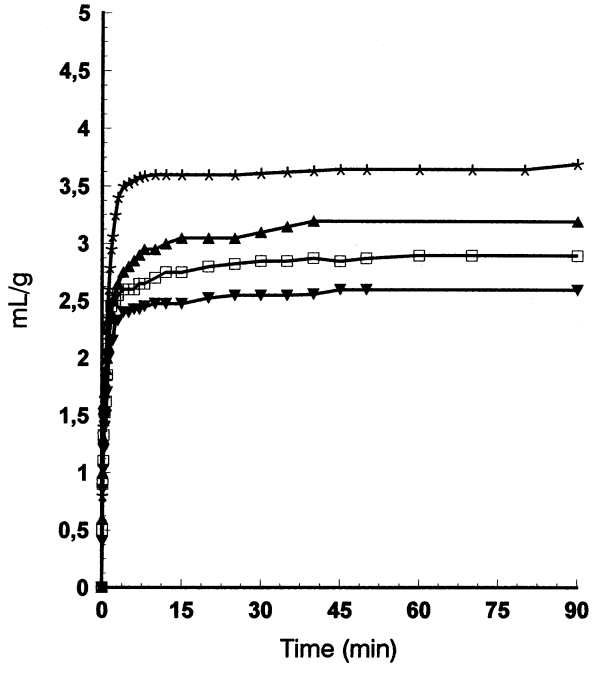

Fig. 2. Volume of water absorbed (ml/g) of gelatin microspheres crosslinked D,L-glyceraldehyde at 37°C; ★ gelatin microspheres crosslinked with 0.5% (w/v) of D,L-glyceraldehyde for 24 h; □ gelatin microspheres crosslinked with 1% (w/v) of D,L-glyceraldehyde for 24 h; ▲ gelatin microspheres crosslinked with 2% (w/v) of D,L-glyceraldehyde for 1 h; ▼ gelatin microspheres crosslinked with 2% (w/v) of D,L-glyceraldehyde for 24 h.

Table 3
Equilibrium swelling values (ml/g) of the different crosslinked gelating microsphere samples[a]

Gelatin microspheres	Equilibrium swelling values (ml/g)
Crosslinked with 0.5% of GAL for 24 h	3.7 (0.2)
Crosslinked with 1% of GAL for 24 h	2.9 (0.2)
Crosslinked with 2% of GAL for 24 h	2.6 (0.1)
Crosslinked with 2% of GAL for 1 h	3.2 (0.1)

[a] S.E. in parentheses.

is reported in Fig. 3 as a function of time. Under the condition used in the 'in vitro' study a biphasic pattern of drug release was evident for all the samples. Thus, independently of the conditions of gelatin microsphere crosslinking, in all cases there was a an initial short period of rapid drug release followed by a second, more prolonged, period during which slower drug release took place. Increasing the crosslinker concentration the amount of drug released in the first minutes, from the microsphere crosslinked for 24 h, decreased from about 60 to 30%. Changing the crosslinking time period from 24 to 1 h, the drug immediately released from the microspheres crosslinked with higher concentration of GAL increased from about 29 to about 40%. In the second phase, practically all the drug was released from the

Table 4
Drug content in the different crosslinked gelatin microsphere samples[a]

Gelatin microspheres	Clonidine hydrochloride (mg/100 mg)
Crosslinked with 0.5% of GAL for 24 h	4.2 (0.2)
Crosslinked with 1% of GAL for 24 h	2.9 (0.1)
Crosslinked with 2% of GAL for 24 h	2.8 (0.1)
Crosslinked with 2% of GAL for 1 h	3.0 (0.1)

[a] S.E. in parentheses.

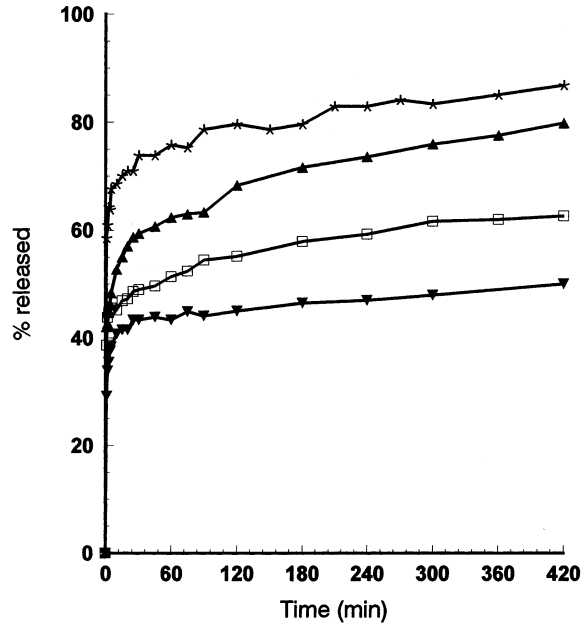

Fig. 3. 'In vitro' clonidine hidrocloride release from gelatin microspheres crosslinked D,L-glyceraldehyde; ★ gelatin microspheres crosslinked with 0.5% (w/v) of D,L-glyceraldehyde for 24 h; □ gelatin microspheres crosslinked with 1% (w/v) of D,L-glyceraldehyde for 24 h; ▲ gelatin microspheres crosslinked with 2% (w/v) of D,L-glyceraldehyde for 1 h; ▼ gelatin microspheres crosslinked with 2% (w/v) of D,L-glyceraldehyde for 24 h.

microsphere crosslinked with either the lowest concentration of crosslinker or the lowest crosslinking time period, whereas only the 60 or 50% of clonidine was checked in the medium from the microsphere crosslinked with 1 or 2% (w/v) of GAL, respectively. After the experimental time considered, clonidine hydrochloride was released very slowly and the full drug release was approached only after some days for both the samples. In conclusion, the 'in vitro' studies carried out with gelatin microspheres prepared using various GAL concentration or different crosslinking time periods indicate that as the crosslinking extent increases a corresponding decrease of drug release rate is achieved. This effect can be related to the different crosslinking degree of the gelatin microspheres, which affected both the water penetration, and hence the drug diffusion, and swelling capability of the microsphere sample.

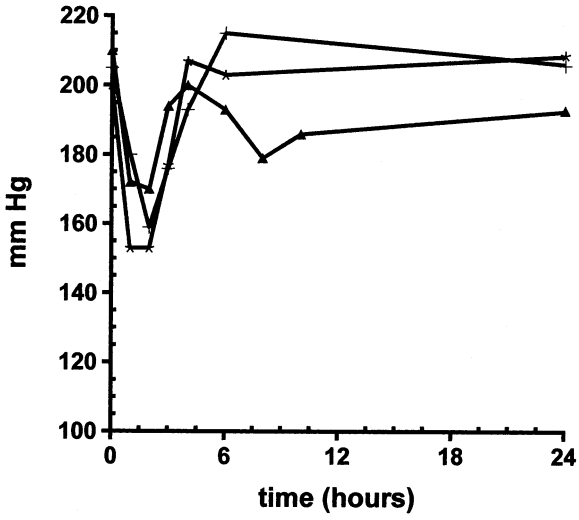

Fig. 4. Mean SBP after the administration of, + reference solution; * gelatin microspheres crosslinked with 0.5% (w/v) of D,L-glyceraldehyde for 24 h; ▲ gelatin microspheres crosslinked with 2% (w/v) of D,L-glyceraldehyde for 1 h.

3.7. Antihypertensive effect studies

To verify the capability of gelatin microspheres crosslinked with GAL to prolong in vivo the duration of the drug effect, the antihypertensive effect of clonidine hydrochloride released from the microspheres was studied. Practically, the effect on the SBP obtained after the subcutaneous injection of the microspheres was recorded and compared with that of a clonidine hydrochloride solution containing the same drug amount. No effect on the blood pressure was noticed after administration of unloaded microspheres. With the loaded microspheres crosslinked either with the lowest concentration of GAL (MS 0.5%; 24 h) or with the shortest time period (MS 2%; 1 h), there was a rapid fall in SBP which peaked 1–2 h after the administration as with the reference solution of clonidine hydrochloride (Fig. 4). Then, the SBP increased reaching the basal value in 4–6 h both with the microspheres crosslinked with the lowest concentration of GAL (MS 0.5%; 24 h) and with the reference solution, whereas a statistically significant decrease of the blood pressure of about 20 mmHg was still recorded 8–24 h with the loaded microspheres crosslinked for the shortest time period (MS 2%; 1 h) (Fig. 4). Really with this microsphere sample (Table 5), a biphasic profile in the SBP lowering has been pointed out, i.e. after the peak the SBP increased reaching the basal value in 4 h and then was still lowered until 24 h. After the subcutaneous injection of the loaded microspheres crosslinked for 24 h with concentration of GAL higher than 0.5% (w/v), a more gradual and sustained SBP reduction was

Table 5
Mean systolic pressure values (mmHg) obtained before and after the treatment either with reference solution or with different gelatin microsphere sample[a]

Time (h)	Reference solution	MS 2% 1 h	MS 2% 1 h	MS 1% 24 h	MS 1% 24 h
0	205 (5)	200 (5)	210 (9)	195 (10)	206 (15)
1	180 (3)*	153 (11)*	172 (14)*	170 (19)**	192 (16)
2	159 (15)*	153 (4)*	170 (18)*	162 (16)*	188 (7)***
3	176 (18)*	177 (17)***	194 (16)**	166 (27)***	181 (9)**
4	193 (27)**	207 (17)	200 (14)	166 (16)**	182 (10)**
6	215 (10)	203 (203)	193 (20)	160 (29)**	179 (18)**
8			179 (15)*	159 (24)**	172 (17)**
10			186 (23)***	163 (19)*	160 (15)*
24			193 (23)***	155 (18)*	158 (17)*
28			205 (8)	145 (20)*	148 (15)*
32				161 (23)**	158 (12)*
48				170 (14)**	147 (12)*
52				170 (18)**	155 (14)*
72				188 (18)	185 (20)

[a] *$P<0.001$ versus baseline; **$P<0.05$ versus baseline; ***$P<0.01$ vs. baseline; S.E. in parentheses.

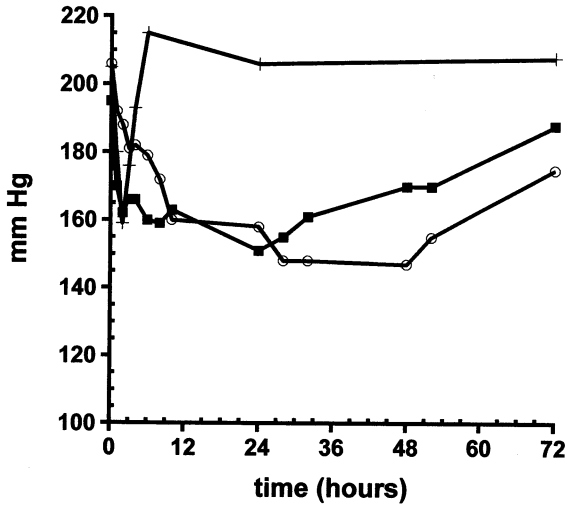

Fig. 5. Mean SBP after the administration of, + reference solution; ■ gelatin microspheres crosslinked with 1% (w/v) of D,L-glyceraldehyde for 24 h; ○ gelatin microspheres crosslinked with 2% (w/v) of D,L-glyceraldehyde for 24 h.

showed (Fig. 5). The maximum SBP lowering was achieved 2 h post injection, as with the reference solution, in the case of loaded microspheres crosslinked with 1% (w/v) of GAL, whereas only about 10 h postdose with those crosslinked with 2% (w/v) (Table 5). Then, the antihypertensive effect was maintained until 52–72 h after the injection with both the microspheres samples (Table 5). These results could be clearly explained by the different extent of crosslinking of the microspheres, i.e. by the different control on the drug release of the gelatin matrix. According to the 'in vitro' release profiles, the initial rapid fall of SBP could be the consequence of the initial short period of rapid drug release. The subsequent gradual release showed in the 'vitro' profiles (Fig. 3) justified the prolongation of 'in vivo' effect observed (Fig. 5). The in 'vivo' results allow the conclusion to be drawn that gelatin microspheres crosslinked with 1 or 2% (w/v) of D,L- glyceraldheide for 24 h appear to be able to prolong the antihypertensive effect of clonidine hydrochloride after subcutaneous administration.

3.8. Biocompatibility studies

Histological sections of subcutaneous tissue at the injection site of sterilised gelatin microspheres (crosslinked with 2% (w/v) of GAL for 24 h) are shown in Fig. 6. Up to 24 h post injection, the microspheres were still discerned in the tissue and a weak inflammatory response characterised by the presence of eosinophils and polymorphs was clearly evident (Fig. 6b). Then, the tissue response progressed from the initial inflammation to a chronic inflammatory response, characterised by the presence of macrophages and foreign body giant cells around the microspheres that are to be bioeroded (Fig. 6c). No microspheres or their fragments were detected at 3 months after the injection suggesting their complete biodegradation. The inflammation of the tissue observed until 1 month after the injection could be considered as a typical reaction produced by a foreign body, which is completely resolved after the mi-

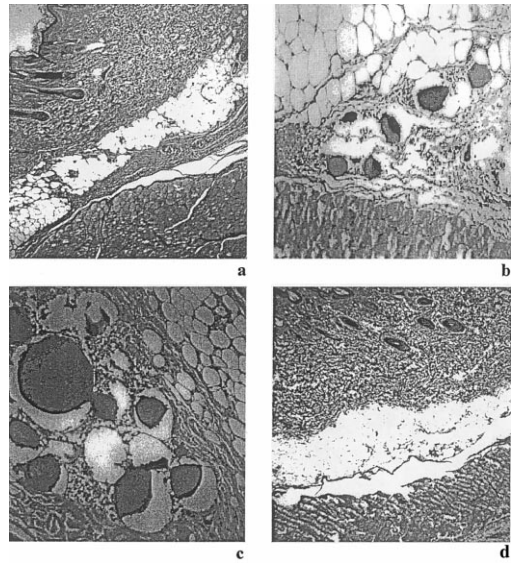

Fig. 6. Light photomicrographs of subcutaneous injection site tissue of, (a) controls and animals which received microspheres up to 6 months (original magnification × 25); (b) animals which received microspheres 24 h post injection (original magnification × 100); (c) animals which received microspheres 1 month after the injection (original magnification × 200); (d) animals which received microspheres 3 months after the injection (original magnification × 25)

crosphere biodegradation. In fact, the tissue returned to normal appearance within 3 months (Fig. 6d). Moreover, no adverse reactions (calcification, necrosis, tumorigenesis and infection) were observed at the implantation site up to 6 months. These findings clearly indicate that the gelatin microspheres crosslinked with GAL are well tolerated in vivo.

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