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Pharmacological studies on frozen stored canine saphenous veins and basilar arteries

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Summary. Canine saphenous veins were either placed in Krebs-Henseleit solution and stored for 24 h at +4°C, or immersed in FCS (fetal calf serum) containing 1.8 mol/l DMSO (dimethyl sulfoxide), slowly frozen to −70°C and stored for 4 weeks at −70°C or −190°C. Canine basilar arteries were either stored in Krebs-Henseleit solution for 24 h at +4°C or slowly frozen and stored for 3 months in FCS plus 1.8 mol/l DMSO at −70°C. Subsequent pharmacological investigations revealed a considerable attenuation of the contractile force of frozen-stored vessels but the evidence suggests that there may be a very good preservation of the main biochemical properties, such as monoamine oxidase activity, endogenous prostaglandin synthesis and uptake$_1$ mechanisms in veins stored at −190°C and there is an excellent correlation of the pD_2 values for various tryptamine derivatives on canine basilar arteries stored for 3 months at −70°C with those calculated on fresh preparations. It is concluded that freezing isolated blood vessels may be considered an effective means of preserving and storing vascular tissues for pharmacological investigations.

Key words: Cryopreservation – Saphenous veins – Basilar arteries – DMSO – Vessel bank

Introduction

Pharmacological studies on isolated blood vessels generally require freshly obtained vascular tissue. Eventually vessels stored for some hours in physiological salt solution at +4°C can be used, but this makes it necessary to kill an animal from which more vascular material can be obtained than can be used within the available time. Thus the advantages of a simple, reliable storage method for ensuring the availability of adequate vascular preparations for pharmacological studies are readily apparent. Creation of a 'vessel bank' for pharmacological studies requires that the storage method preserves not only the histological structures but also the functional ultrastructure, i.e., the biochemical and mechanical behaviour of the blood vessel employed. In view of the great importance of having isolated blood vessels easily available for pharmacological investigations, we studied the pharmacological behaviour of spiral strips from canine saphenous veins and basilar arteries after different storage methods. Our investigations were encouraged by reports on successful cryopreservation of living mammalian embryos (Elliott and Whelan 1977) and venous and arterial allografts (Balderman et al. 1984; Boren et al. 1978; Stanley et al. 1975; Weber et al. 1975, 1976).

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Materials and methods

Saphenous veins and basilar arteries were obtained from beagle dogs of either sex weighing 7.0–13.2 kg, which had served as controls in toxicological investigations. The dogs were killed by i.v. injection of pentobarbitone (50 mg/kg) and exsanguination from the femoral arteries. The blood vessels were carefully removed, placed in ice-cold Krebs-Henseleit solution (mmol/l: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, EDTA 0.03) gassed with 5% CO_2 in oxygen and transported to the laboratory within 1–4 h. Thereafter the blood vessels were distributed into different groups for various storage methods.

Storage methods. Saphenous veins from 10 dogs were distributed randomly into 4 groups of 5 veins. Group I consisted of 'fresh veins' which were used immediately after removal for organ bath studies. The veins in group II were stored for 24 h in Krebs-Henseleit solution at +4°C before use. Each vein of group III and IV was first perfused with FCS (fetal calf serum) containing 1.8 mol/l DMSO (dimethyl sulfoxide) as a cryoprotecting agent, before being immersed in 2 ml Liquid Nitrogen Ampoules (Gibco AG, Basel, Switzerland) containing the same solution. The ampoules were then placed in a styropor box (11 × 11 × 22 cm) and slowly frozen in a freezer maintained at −70°C. The 5 veins in group III were stored for 4 weeks at −70°C, while those in group IV were transferred after 24 h into liquid nitrogen (−190°C) where they were stored for 4 weeks. Before thawing, the veins of group IV were placed again for 1–20 h in the freezer maintained at −70°C. Prior to the pharmacological investigation the veins of group III and IV were thawed within 2 min by placing the ampoules in a +37°C water bath.

Basilar arteries: The basilar arteries were distributed into two groups. Group I consisted of arteries which were used either immediately or 24 h after removal and storage in Krebs-Henseleit solution at +4°C. Group II consisted of 10 arteries which were treated and frozen as described for group III of the saphenous veins but without perfusion of the vessel.

Experimental procedures. The veins were cut into helical strips (15 × 2 mm) and suspended in 10 ml organ baths

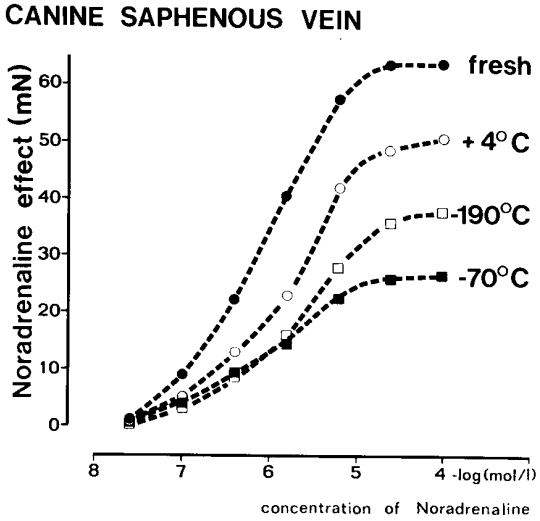

Fig. 1. Concentration-response curves for noradrenaline on canine saphenous vein strips after different storage procedures. Vein strips were investigated immediately after removal (fresh), after 24 h storage in Krebs-Henseleit solution at +4° C (+4° C), or after 3 weeks storage in FCS plus 1.8 mol/l DMSO at −70° C (−70° C) and in liquid nitrogen (−190° C). The curves were constructed from the mean responses to noradrenaline of the respective control strips from each group and converted into mN according to the data given in Table 1

containing Krebs-Henseleit solution at +37° C, gassed continuously with 5% CO_2 in O_2. Changes in the tone of the preparations were monitored isometrically. At the beginning of the experiments the venous strips were stretched to an initial tension of 7.5 mN and allowed to establish baseline tension and to equilibrate for at least 4 h in the bathing medium, which was changed every 15 min. During this time the preparations were exposed twice to noradrenaline (10 µmol/l) before the maximum response to noradrenaline was determined by cumulative additions. This effect was taken as point of comparison for subsequent responses. After repeated washouts cumulative concentration-response curves for 5-HT and noradrenaline were determined in the presence and absence of cocaine (30 µmol/l), aspirin (30 µmol/l) or indomethacin (300 nmol/l) and curves for tryptamine were determined in the presence and absence of pargyline (30 µmol/l). In each experiment 6 strips from the same vein were investigated at the same time. Inhibitors were added 20 min before the agonists to the organ baths and remained in contact with the organ when the responses to agonists were tested.

The arterial strips (10 × 1 mm) were stretched to an initial tension of 5 mN and allowed to equilibrate for 2 − 3 h in the bathing medium. Cumulative concentration-response curves for 5-HT and various tryptamine derivatives were determined in the presence of 30 µmol/l pargyline added 20 min before the agonist in order to prevent deamination of the indole derivatives by monoamine oxidase (Vane 1959). In each experiment 2 or 3 cumulative concentration-response curves for 5-HT were first determined until the reproducibility of the effects indicated a consistent response by the arterial strips. Responses to the agonists were expressed as a percentage of the preceding maximal response to 5-HT. In each experiment 6 strips of the same artery were investigated at the same time, one of which was used as a control preparation, to correct for any sensitivity changes during the experiment.

Drugs. The following pharmacological agents were used: (−)-noradrenaline hydrogen tartrate (Hoechst, Frankfurt/Main, FRG), 5-hydroxytryptamine creatinine sulfate (5-HT), 5-methoxytryptamine hydrochloride (5-OCH_3-T, Fluka, Buchs, Switzerland), cocaine hydrochloride (Lehner, Muttenz, Switzerland), indomethacin (Merck and Co., Inc., Darmstadt, FRG), 5-methyltryptamine (5-CH_3-T), tryptamine hydrochloride (T), pargyline hydrochloride (Sigma, Munich, FRG), alpha-methyl-5-hydroxytryptamine creatinine sulfate (α-CH_3-5-HT, Upjohn, Kalamazoo, MI, USA), beta-methyl-5-hydroxytryptamine hydrogen oxalate (β-CH_3-5-HT), omega-N-methyl-5-hydroxytryptamine oxalate (ω-N-CH_3-5-HT), N,N-dimethyl-5-hydroxytryptamine binoxalate [N,N-$(CH_3)_2$-5-HT], 5-aminotryptamine oxalate (5-NH_2-T), 4-hydroxytryptamine creatinine sulfate (4-HT), acetylsalicylic acid (Sandoz Ltd., Basel, Switzerland), dimethyl sulfoxide (DMSO, Merck-Schuchardt, Hohenbrunn, FRG). All compounds were dissolved just before use. Drug concentrations are given as molar concentrations throughout. Statistical analysis of data was performed using Student's *t*-test.

Results

Canine saphenous veins

Baseline tension. When the initial tension of 7.5 mN had been applied, the mean baseline tone established by the saphenous vein strips was 1.78 ± 0.10 mN (mean ± SEM, $n = 30$) in the group of fresh veins, 1.66 ± 0.11 mN (mean ± SEM, $n = 30$) in the group of veins stored for 24 h at +4° C and 1.79 ± 0.14 mN (mean ± SEM, $n = 30$) in the group of veins stored for 4 weeks at −190° C, the values being statistically not different. By contrast, strips prepared from veins stored for 4 weeks at −70° C relaxed to a mean baseline tone of 2.47 ± 0.25 mN (mean ± SEM, $n = 30$) which was significantly higher ($p < 0.001$) than those observed with the former 3 groups.

Contractile force. The contractile force as assessed by the maximal response in mN of vein strips to noradrenaline, varied significantly between the 4 groups. Compared to the maximum response to noradrenaline of fresh veins, storage for 24 h at +4° C diminished the contractile force of the vein strips to about 80%. After freezing for 24 h to −70° C and storage for 4 weeks at −190° C the force developed by the vein strips was still about 60%, while storage for 4 weeks at −70° C attenuated the contractile force to about 40% of that produced by fresh veins (Fig. 1). Additional experiments have shown that freezing directly in liquid nitrogen or storage of veins for 24 − 72 h at −20° C before placing them into liquid nitrogen markedly attenuated or even abolished completely the contractile responsiveness of the venous preparations. The maximum response to 5-HT, expressed as percentage of the maximal noradrenaline effect, for each condition declined with decreasing storage temperatures. Compared to that of fresh veins, the maximal response to 5-HT was depressed to 90% after storage for 24 h at +4° C and diminished further to 67% and 55% after storage for 4 weeks at −70° C and −190° C respectively. By

Table 1. Maximal contractile responses of fresh and stored canine saphenous veins expressed as a percentage of the maximum noradrenaline effect. Maximal contractile response to noradrenaline (NA) in mN and to NA and 5-HT in the presence and absence of cocaine (30 μmol/l), aspirin (30 μmol/l) or indomethacin (300 nmol/l) and to tryptamine in the presence and absence of pargyline (30 μmol/l) relative to the maximal NA effect on canine saphenous vein strips after different storage methods. Inhibitors were added 20 min before the agonists, data are given as means ± SEM, for each $n = 5$, for NA in mN $n = 30$

	Fresh vein strips		Stored for 24 h at +4° C		Stored for 4 weeks at −70° C		Stored for 4 week at −190° C	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
NA (mN)	64.3	(5.2)	50.9	(3.9)	26.7	(1.9)	38.0	(2.6)
NA (%)								
Control	100.0		100.0		100.0		100.0	
+ Cocaine	95.2	(4.4)	100.6	(2.2)	104.8	(2.6)	100.4	(3.4)
+ Aspirin	95.4	(3.1)	98.2	(2.6)	100.8	(3.9)	102.0	(1.9)
+ Indomethacin	98.8	(1.4)	101.0	(2.6)	99.2	(4.1)	103.2	(3.8)
5-HT (%)								
Control	50.8	(4.7)	46.4	(3.9)	33.8	(3.8)	27.4	(0.8)
+ Cocaine	50.6	(6.2)	43.0	(4.0)	35.0	(1.1)	30.0	(3.5)
+ Aspirin	40.0	(6.0)	36.2	(3.9)	32.0	(3.6)	23.8	(3.1)
+ Indomethacin	36.0	(3.0)*	33.4	(2.7)*	31.0	(2.7)	21.4	(2.8)*
Tryptamine (%)								
Control	75.7	(1.2)	67.4	(2.8)	78.6	(4.3)	72.2	(5.6)
+ Pargyline	77.6	(4.7)	66.4	(7.0)	66.4	(4.2)*	73.6	(6.7)

Significant differences compared to the respective control values are * = $p < 0.05$

Table 2. pD_2 values on fresh and stored canine saphenous veins. pD_2 values for noradrenaline (NA) and 5-HT in the presence and absence of cocaine (30 μmol/l), aspirin (30 μmol/l) or indomethacin (300 nmol/l) and for tryptamine in the presence and absence of pargyline (30 μmol/l) on canine saphenous vein strips after different storage methods. Inhibitors were added 20 min before the agonists, data are given as means ± SEM, for each $n = 5$

	Fresh vein strips		Stored for 24 h at +4° C		Stored for 4 weeks at −70° C		Stored for 4 weeks at −190° C	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
NA								
Control	6.06	(0.07)	5.86	(0.10)	5.95	(0.14)	5.67	(0.06)
+ Cocaine	7.11	(0.15)***	6.88	(0.09)***	6.82	(0.06)***	6.74	(0.03)***
+ Aspirin	5.84	(0.12)	5.69	(0.10)	5.84	(0.13)	5.57	(0.03)
+ Indomethacin	5.89	(0.12)	5.63	(0.08)	5.92	(0.09)	5.78	(0.11)
5-HT								
Control	6.68	(0.10)	6.50	(0.07)	6.48	(0.09)	6.46	(0.08)
+ Cocaine	7.28	(0.12)**	7.04	(0.08)***	6.91	(0.10)*	7.00	(0.07)***
+ Aspirin	6.47	(0.11)	6.37	(0.06)	6.44	(0.09)	6.24	(0.09)*
+ Indomethacin	6.51	(0.09)	6.31	(0.03)*	6.56	(0.11)	6.33	(0.09)
Tryptamine								
Control	4.23	(0.08)	4.33	(0.19)	4.38	(0.15)	4.27	(0.06)
+ Pargyline	5.25	(0.14)***	5.29	(0.05)**	5.36	(0.09)***	5.31	(0.10)***

Significant differences compared to the respective control values are * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

contrast, the maximum contractile effect of tryptamine in comparison to noradrenaline was similar in all groups, i.e., the storage-induced changes of the maximal responses to tryptamine paralleled those of noradrenaline (Table 1).

pD_2 values of agonists. Compared to the group of fresh veins the pD_2 values of both noradrenaline and 5-HT were slightly diminished in all groups of stored veins but only in the group of veins stored for 4 weeks at −190° C did the reduction of the pD_2 value of noradrenaline reach significance ($p < 0.005$). By contrast, the pD_2 values of tryptamine were identical in the 4 groups (Table 2).

$Uptake_1$ mechanism. In each group treatment of the saphenous vein strips with cocaine (30 μmol/l) shifted the concentration-response curves for both noradrenaline and 5-HT to the left thereby increasing the pD_2 values significantly. The enhancement of the venoconstrictor response to 5-HT by cocaine was somewhat less pronounced in the group of veins stored for 4 weeks at −70° C as compared to the other 3 groups.

Blockade of endogenous prostaglandin synthesis. Neither aspirin (30 μmol/l) nor indomethacin (300 nmol/l) modified the constrictor responses to noradrenaline in any group,

Table 3. Effects of tryptamine derivatives on canine basilar arteries without and after storage at −70° C. pD_2 values and maximal contractile effects (E_{max}) relative to 5-HT of various tryptamine derivatives on canine basilar arteries stored for 3 months at −70° C compared to values determined on fresh preparations. Data are given as means ± SEM, for each $n = 5$, for 5-HT $n = 15$

Tryptamine derivative	Fresh basilar artery				Stored basilar artery			
	pD_2	± SEM	E_{max}	± SEM	pD_2	± SEM	E_{max}	± SEM
1. 5-HT	7.02	0.06	1.00	0.06	7.27	0.08	1.00	0.03
2. 5-CH_3-T	6.75	0.11	1.18	0.08	7.08	0.05	1.11	0.08
3. 5-OCH_3-T	7.20	0.10	1.19	0.05[a]	7.18	0.04	1.01	0.04
4. α-CH_3-5-HT	6.47	0.17	0.90	0.08[a]	6.46	0.06	0.97	0.02
5. β-CH_3-5-HT	5.83	0.17	0.64	0.05	6.05	0.03	0.87	0.04
6. ω-N-CH_3-5-HT	7.18	0.10	0.82	0.10[a]	7.45	0.06	0.81	0.06
7. $NN(CH_3)_2$-5-HT	6.86	0.08	0.74	0.09	7.02	0.19	0.92	0.10
8. 5-NH_2-T	6.33	0.20	0.92	0.04[a]	6.40	0.12	1.07	0.07
9. 4-HT	6.37	0.10	1.25	0.09[a]	6.71	0.13	0.86	0.05
10. Tryptamine	6.42	0.07	1.07	0.08[a]	6.35	0.13	0.91	0.03

[a] Data taken from Müller-Schweinitzer and Engel (1983)

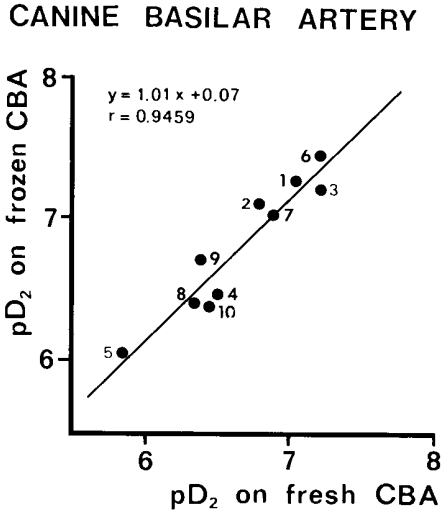

Fig. 2. Correlation between pD_2 values of tryptamine derivatives determined in the presence of 30 µmol/l pargyline on spiral strips from canine basilar arteries (CBA) stored for 3 months at −70°C and of those determined on fresh preparations. The data, taken from Table 3, were compared by linear regression analysis and the correlation coefficient is given in the figure

but both compounds diminished the maximal responses to 5-HT. This effect again was only marginal and did not reach significance level in the group of veins stored for 4 weeks at −70°C.

Blockade of MAO activity. In each of the 4 vein groups blockade of the MAO activity by pargyline (30 µmol/l) shifted the concentration-response curve for tryptamine to the left, thereby increasing the pD_2 value of the agonist significantly by a factor of about 10.

Canine basilar arteries

The responsiveness to various tryptamine derivatives of canine basilar arteries was studied after a storage time of the preparations of 98 ± 12 days (mean ± SEM, $n = 9$) at −70°C. When the initial tension of 5.0 mN had been applied, strips from fresh basilar arteries relaxed to a baseline tension of 1.95 ± 0.05 mN (mean ± SEM, $n = 60$) while strips prepared from arteries stored for 3 months at −70°C established a significantly higher baseline tension of 2.71 ± 0.21 mN (mean ± SEM, $n = 60$, $p < 0.0005$). Compared to the maximal contraction in response to 5-HT elicited by strips from fresh canine basilar arteries (1.49 ± 0.15 mN, mean ± SEM, $n = 60$) the contractile response to 5-HT of frozen-stored arteries (1.03 ± 0.10 mN, mean ± SEM, $n = 60$) was reduced to 69% ($p < 0.005$). However, despite of the attenuated contractility, the tryptamine derivatives stimulated basilar arteries of both groups with similar pD_2 values (Table 3). As shown in Fig. 2, the pD_2 values of the tryptamine derivatives calculated on frozen-stored basilar arteries correlate very well with those calculated on fresh preparations ($r = 0.95$, $p < 0.001$).

Discussion

When living mammalian cells are frozen in physiological media without additives, generally only few or even no cells survive due to injury caused by the formation of ice crystals in intracellular and/or extracellular spaces (Litvan 1972; Mazur 1977). Besides a protein-rich medium such as serum, the low molecular weight compound DMSO has been shown to protect successfully vascular tissues from cryoinjury during freezing. Thus conservation of canine venous homografts at < −120°C in a 15% DMSO solution for several weeks yielded not only acceptable long-term patency with low rate of thrombosis but also satisfactory histological characteristics, e.g., a high rate of intact endothelium (Balderman et al. 1984; Boren et al. 1978; Malone et al. 1980; Weber et al. 1975, 1976), intact smooth muscle cells and unchanged elastic properties (Weber et al. 1975; L'Italien et al. 1979). On the other hand, in the high concentrations necessary for cryoprotection, DMSO itself is known to cause considerable non-specific damage to smooth muscle cells (Farrant 1964). In contraction studies on canine jugular veins Weber et al. (1975) have demonstrated that incubation of venous preparations for 1 h at room temperature in physiological salt solution (PSS) containing 20% and 25% DMSO (= 2.8 and 3.5 mol/l) significantly attenuated the maximal responses to adrenaline to 40% and 20% respec-

tively, while 15% DMSO (= 2.1 mol/l) diminished the contractile response to adrenaline (5 μg/ml) only to about 75%. Therefore in the present series of validation studies of preservation of isolated blood vessels, canine saphenous veins and basilar arteries were immersed in FCS (fetal calf serum) containing 1.8 mol/l DMSO as a cryoprotecting agent. It has been shown, furthermore, that for the survival of the endothelium during extracorporeal maintenance of a vessel, besides a protein-rich perfusate relaxation of the vascular smooth muscle is important (Haudenschild et al. 1981). In those studies papaverine (158 nmol/l) was applied to achieve this condition. Since, however, DMSO itself causes complete relaxation of smooth muscle when used at concentrations of 2.0 mol/l and above (Jackson et al. 1979; Spilker 1972) we prepared our medium without any additional smooth muscle relaxant. The results of our pharmacological experiments with frozen stored canine saphenous veins demonstrate, that freezing of this vascular tissue in FCS plus 1.8 mol/l DMSO preserves uptake$_1$ mechanisms, endogenous prostaglandin synthesis and monoamine oxidase activity though the contractile force in response to both noradrenaline and 5-HT was considerably attenuated after storage at temperatures of −70° C and below. The attenuation of the maximal response to noradrenaline of the veins stored at −190° C to about 60% of that produced by fresh veins is in accordance with the reduction of the contractile force of DMSO-treated frozen canine jugular veins observed by Dent et al. (1974) and Weber et al. (1975) when they tested the responsiveness of their allografts by exposing the preparations to a single submaximal concentration of adrenaline. Relative to noradrenaline, the contractile response to 5-HT was considerably more susceptible to freezing procedures than was that of tryptamine. These observations strongly suggest that 5-HT and tryptamine stimulate canine saphenous veins through separate receptors and/or mechanisms. Analysis of this contention will be subject of further investigations. According to our present results, slow freezing to −70° C and storage at −190° C in FCS plus 1.8 mol/l DMSO yields better cryopreservation than storage at −70° C.

A similar reduction of the contractility as in veins was observed in canine basilar arteries after storage for 3 months in FCS plus 1.8 mol/l DMSO at −70° C. In these preparations the contractile force was attenuated to about 70% compared to that of fresh basilar arteries. But despite this diminution of the contractile force, there was an excellent correlation of the pD_2 values for various tryptamine derivatives on frozen stored basilar arteries compared to the pD_2 values calculated on fresh preparations, suggesting that the conformation of the 5-HT receptor was maintained undamaged.

In conclusion, our present results suggest that despite an attenuated contractile force observed with DMSO-treated frozen stored canine blood vessels, the main biochemical properties of the tissues are maintained. In general, immersion of canine saphenous veins in FCS containing 1.8 mol/l DMSO, slow freezing to −70° C and storage in liquid nitrogen at −190° C yielded a better cryopreservation than storage at −70° C. Freezing of isolated blood vessels may thus be considered an effective means of preserving and storing vascular tissues for pharmacological investigations.

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