Biological and Hemodynamic Effects of Low Doses of Fludrocortisone and Hydrocortisone, Alone or in Combination, in Healthy Volunteers With Hypoaldosteronism

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Low doses of hydrocortisone (HC) and fludrocortisone (FC) administered together improve the prognosis after septic shock; however, there continues to be disagreement about the utility of FC for this indication. The biological and hemodynamic effects of HC (50 mg intravenously) and FC (50 μg orally) were assessed in 12 healthy male volunteers with saline-induced hypoaldosteronism in a placebo-controlled, randomized, double-blind, crossover study performed according to a 2 × 2 factorial design. HC and FC significantly decreased urinary sodium and potassium levels (from −58% at 4 h to −28% at 10 h and from −35% at 8 h to −24% at 12 h, respectively) with additive effects. At 4 h after administration, HC significantly increased cardiac output (+14%), decreased systemic vascular resistances (−14%), and slightly increased heart rate (+4 beats/min), whereas FC had no hemodynamic effect. At doses used in septic shock, HC induced greater mineralocorticoid effect than FC did. HC also induced transient systemic hemodynamic effects, whereas FC did not. New studies are required to better define the optimal dose of FC in septic shock.

Septic shock is frequently associated with primary adrenal insufficiency.[1,2] To evaluate the efficacy of hormone replacement therapy for this indication, we previously conducted a placebo-controlled, randomized, double-blind trial to test the effects of physiologic doses of a combination of hydrocortisone (HC), 50 mg intravenously four times daily, and fludrocortisone (FC), 50 μg orally once daily. That trial demonstrated that these low doses of steroids improved the prognosis in patients with vasopressor-unresponsive septic shock, especially in those with relative adrenal insufficiency.[3] These results were not replicated in another trial that assessed the efficacy of HC alone, 50 mg intravenously four times daily, in nonrefractory septic shock.[4] This raised a debate on the need for dual glucocorticoid and mineralocorticoid supplementation, and there currently is a controversy about whether FC should be coadministered with HC.[5,6] There are several reasons for this hesitation. First, low doses of HC alone have repeatedly been shown to improve vascular responsiveness to catecholamines[7,8] and to decrease time to shock reversal;[9–12] and two meta-analyses confirmed that the administration of HC improves patient survival rates.[13,14] Similar data do not exist for FC alone. Second, at the doses used in septic shock, HC could provide adequate mineralocorticoid activity, rendering the addition of FC superfluous.[15] Third, there is doubt about the bioavailability of orally administered FC in patients with septic shock, who commonly have impaired hepatosplanchnic perfusion.[16–18] Fourth, the biological and hemodynamic effects of each drug, alone or in combination, have not been evaluated at the doses used in septic shock. Fifth, in a recent trial to assess the respective roles of intensive insulin therapy and FC (50 μg orally once daily) in patients with septic shock who were receiving treatment with HC, FC did

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not induce any statistically significant improvement in patient outcomes, thereby casting further uncertainty on its applicability in this context.[19] Therefore, there is a need for clarification, especially about the relative contributions of each drug to mineralocorticoid effects. We investigated the biological and hemodynamic effects of single administration of low doses of HC and FC, given either alone or in combination, in healthy volunteers. To mimic the relative adrenal insufficiency frequently observed in septic shock, we used a model of hypoaldosteronism induced by intravenous sodium loading.

RESULTS

Baseline biological and hemodynamic variables

There were no significant differences at baseline, in any of the biological and hemodynamic variables, between the four periods of investigation (**Table 1**).

Effects on biological variables

Plasma levels of aldosterone and renin decreased after saline infusion (−63 and −41%, respectively), reflecting the expected hypoaldosteronism induced by sodium loading.

Table 2 shows the changes in levels, with time, of plasma sodium, potassium, and glucose. A significant FC effect was observed in relation to sodium level ($P = 0.003$). Time-point comparisons showed that FC slightly increased sodium at T8 (+1 mmol/l). However, all values stayed within the normal range (135–145 mmol/l), and this variation, although statistically significant, had no clinical consequence. No significant effects of either FC or HC were found with respect to potassium level. HC was observed to have a significant effect on glucose concentration ($P < 0.001$). Time-point comparisons showed that HC significantly increased glucose level at T4 (+11%), T8 (+9%), T12 (+8%), and T24 (+6%). Interestingly, after administration of HC, glucose values were equal to or higher than normal laboratory values (3.9–6.1 mmol/l) at T8 and T12.

Figure 1 shows the changes in urine output with time. A significant FC × HC × time interaction ($P = 0.046$) was observed. Time-point comparisons showed that HC increased urine output between 0 and 1 h (+18%) and between 1 and 4 h (+29%) and that it decreased urine output between 4 and 12 h, the latter effect becoming statistically significant only between 10 and 12 h (−27%). FC also decreased urine output at the time points between 6 and 12 h but not to a significant extent.

Figure 2 shows the changes in urinary sodium/potassium ratio and sodium excretion fraction with time. Both HC and FC produced significant effects in both variables, the effects being

Table 1 Biological and hemodynamic variables at baseline

Variable	Placebo	FC	HC	FC + HC	*P* value
Plasma variables					
Sodium (mmol/l)	141 ± 1	142 ± 1	142 ± 1	142 ± 2	0.512
Potassium (mmol/l)	3.9 ± 0.3	3.9 ± 0.3	3.9 ± 0.3	3.9 ± 0.3	0.994
Glucose (mmol/l)	5.4 ± 0.8	5.6 ± 0.7	5.0 ± 0.5	5.4 ± 0.9	0.097
Creatinine (μmol/l)	90 ± 9	91 ± 9	92 ± 8	92 ± 7	0.452
Aldosterone (pg/ml)	26.8 ± 9.1	24.4 ± 7.8	25.9 ± 9.4	23.5 ± 6.3	0.718
Renin (pg/ml)	5.7 ± 2.8	6.3 ± 2.2	5.9 ± 3.5	6.1 ± 2.6	0.748
Epinephrine (pg/ml)	29.0 ± 10.5	26.8 ± 15.5	30.5 ± 15.4	30.0 ± 10.7	0.814
Norepinephrine (pg/ml)	264 ± 138	209 ± 81	299 ± 108	234 ± 81	0.077
Urinary variables					
Sodium/potassium ratio	4.2 ± 2.1	3.7 ± 1.7	4.3 ± 2.4	4.0 ± 2.0	0.639
Sodium fractional excretion (%)	1.8 ± 0.9	1.7 ± 1.1	1.9 ± 0.9	1.6 ± 1.1	0.812
Hemodynamic variables					
SAP (mm Hg)	122 ± 11	122 ± 11	122 ± 13	119 ± 6	0.697
DAP (mm Hg)	71 ± 7	71 ± 8	68 ± 8	71 ± 7	0.446
MAP (mm Hg)	88 ± 7	88 ± 8	86 ± 8	87 ± 6	0.749
Pulse pressure (mm Hg)	51 ± 9	52 ± 10	54 ± 13	48 ± 6	0.364
Heart rate (beats/min)	71 ± 13	70 ± 12	67 ± 12	70 ± 11	0.342
Cardiac output (l/min)	6.0 ± 1.4	6.2 ± 1.4	6.2 ± 1.7	6.2 ± 1.1	0.874
Stroke volume (ml)	90 ± 19	92 ± 16	90 ± 17	88 ± 15	0.766
SVR (dyne·s/cm^5)	1,221 ± 231	1,181 ± 226	1,220 ± 293	1,212 ± 250	0.862
Carotidofemoral PWV (m/sec)	5.5 ± 0.4	5.5 ± 0.7	5.4 ± 0.7	5.3 ± 0.7	0.586

Data are means ± SD.

DAP, diastolic arterial pressure; FC, fludrocortisone; HC, hydrocortisone; MAP, mean arterial pressure; PWV, pulse wave velocity; SAP, systolic arterial pressure; SVR, systemic vascular resistance.

P values correspond to comparisons between the four groups of treatments.

Table 2 Changes in plasma sodium, potassium, and glucose with time

Variable	Treatment	T1	T4	T8	T12	T24	*P* value		
							FC effect	HC effect	FC × HC interaction
Sodium (mmol/l)	Placebo	141 ± 1	141 ± 1	140 ± 2	140 ± 2	140 ± 1	0.003	0.271	0.073
	FC	140 ± 2	141 ± 2	140 ± 2[a]	140 ± 1	140 ± 1			
	HC	140 ± 2	140 ± 1	139 ± 2	139 ± 2	140 ± 2			
	FC + HC	141 ± 1	140 ± 2	141 ± 1[a]	140 ± 1	142 ± 1			
Potassium (mmol/l)	Placebo	3.9 ± 0.2	4.2 ± 0.3	4.2 ± 0.3	4.3 ± 0.2	3.9 ± 0.2	0.309	0.794	0.886
	FC	3.9 ± 0.2	4.2 ± 0.3	4.1 ± 0.2	4.2 ± 0.2	3.9 ± 0.2			
	HC	4.0 ± 0.3	4.3 ± 0.3	4.2 ± 0.2	4.1 ± 0.2	3.8 ± 0.3			
	FC + HC	4.0 ± 0.3	4.3 ± 0.3	4.2 ± 0.3	4.0 ± 0.2	3.8 ± 0.3			
Glucose (mmol/l)	Placebo	5.2 ± 0.8	5.2 ± 0.2	5.7 ± 0.5	5.9 ± 0.3	5.3 ± 0.9	0.712	<0.001	0.106
	FC	5.6 ± 0.6	5.1 ± 0.4	5.6 ± 0.5	5.8 ± 0.3	4.9 ± 0.9			
	HC	5.1 ± 0.8	5.6 ± 0.3[b]	6.1 ± 0.6[b]	6.2 ± 0.6[b]	5.3 ± 1.0[b]			
	FC + HC	5.6 ± 1.0	5.8 ± 0.3[b]	6.2 ± 0.6[b]	6.4 ± 0.5[b]	5.5 ± 0.9[b]			

Data are means ± SD.

FC, fludrocortisone; HC, hydrocortisone; T1, T4, T8, T12, T24: 1, 4, 8, 12, 24 h after treatment administration.

[a]FC effect compares the two periods during which subjects received FC (FC + HC placebo, FC + HC) with the two periods during which they did not (FC placebo + HC placebo, FC placebo + HC). [b]HC effect compares the two periods during which subjects received HC (FC placebo + HC, FC + HC) with the two periods during which they did not (FC placebo + HC placebo, FC + HC placebo).

additive when the treatments were coadministered (there were no significant treatment interactions). Time-point comparisons showed that HC significantly decreased the sodium/potassium ratio between T4 and T10 (−58% at T4, −57% at T6, −46% at T8, and −28% at T10) and sodium excretion fraction between T4 and T12 (−16% at T4, −36% at T8, and −25% at T12). FC had similar effects but with delayed kinetics and lower intensity. The decrease in the sodium/potassium ratio, which started at T6 (−31%, not significant), attained significance between T8 and T12 (−35% at T8, −38% at T10, and −24% at T12). The decrease in sodium excretion fraction, which started at T4 (−11% at T4 and −24% at T8, not significant), was significant only at T12 (−22%).

Figure 3 shows the changes in the hormone levels of the renin–angiotensin and sympathetic nervous systems with time. No significant treatment effect was observed on plasma aldosterone, renin, epinephrine, and norepinephrine concentrations. All values stayed within the ranges of normal laboratory values.

Effects on hemodynamic variables

Figure 4 shows the changes in systolic, diastolic, and mean blood pressure with time. HC was shown to have a significant effect on diastolic blood pressure ($P = 0.018$) and mean blood pressure ($P = 0.049$). Time-point comparisons showed that the decreases in these variables were significant at T8 (−7% for diastolic blood pressure and −4% for mean blood pressure).

Figure 5 shows the changes in heart rate, stroke volume, cardiac output, and systemic vascular resistances with time. HC was observed to have a significant effect on heart rate ($P = 0.003$), cardiac output ($P = 0.028$), and systemic vascular resistances ($P = 0.002$). Time-point comparisons showed that HC slightly but significantly increased heart rate (+4 beats/min) and cardiac output (+14%) at T4 and that it decreased systemic vascular resistances at T4 (−14%) and T8 (−11%). No significant effects were observed on pulse pressure and carotidofemoral pulse wave velocity (data not shown).

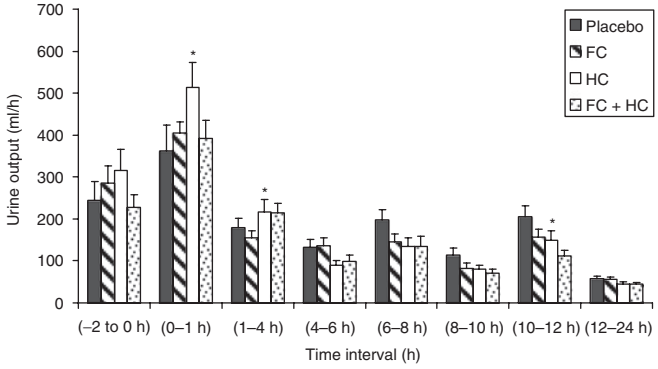

Figure 1 Changes in urine output with time. Data are mean ± SE of the mean. *HC effect compares the two periods during which subjects received HC (FC placebo + HC, FC + HC) with the two periods during which they did not (FC placebo + HC placebo, FC + HC placebo). FC, fludrocortisone; HC, hydrocortisone.

DISCUSSION

Our study investigated the effects of FC and HC, administered either singly or together, using the same single doses of each that are normally administered for septic shock. The study was carried out in the context of the controversy about the utility of FC for this indication. We found that, at the doses used and for the routes of administration employed, a single administration of HC had more marked and earlier mineralocorticoid effects than a single dose of FC; HC also induced systemic hemodynamic effects, whereas FC did not.

Septic shock may be associated with primary adrenal insufficiency, which justifies both glucocorticoid and mineralocorticoid supplementation.[15] This dysfunction of the hypothalamic–pituitary–adrenal axis, also called critical illness–related corticosteroid insufficiency, may affect the balance between pro- and anti-inflammatory pathways and thereby contribute to immune, metabolic, vascular, and organ dysfunctions.[5] The recommended dosage of 200 mg of HC per day (50 mg/4 h) could be sufficient to downregulate the proinflammatory response without causing immune paresis and interfering with wound healing. Therefore, whether there is any additional benefit from the administration of a supplementary mineralocorticoid administration remains to be demonstrated. Furthermore, with respect to mineralocorticoid potency, 20 mg of HC is assumed to be equivalent to 0.05 mg of FC.[15,20] Our study showed that HC had a more rapid and more marked mineralocorticoid activity than FC did, as demonstrated by the greater decrease induced by HC in the urinary sodium/potassium ratio and in sodium fractional excretion, and that the effects were additive. After the administration of HC, the decrease in the sodium/potassium ratio started at T4, was maximal between T4 and T6, and persisted until T10. Interestingly, FC seemed to take the relay baton for HC from T6 onward and to prolong the effect until T12. The differences between HC and FC with respect to the kinetics of effects could possibly result from the different routes of administration of the two treatments, the effect of the intravenously administered drug being observed earlier; thereafter, the orally administered drug begins showing its effects, carrying the baton forward. In practice, in a patient with septic shock, this feature probably has little relevance because HC is administered four times daily, each administration taking the next lap of the relay forward every 6 h, thereby facilitating the maintenance of mineralocorticoid effects.

In our study, urine output started to increase during the first 4 h after HC administration and decreased thereafter. A transient initial increase in urine output after administration of glucocorticoids has previously been described in animal studies; this has been attributed to the ability of glucocorticoids to cause renal vasodilation and to increase glomerular filtration rate.[21] We also observed an increase in blood glucose level after administration of HC, reflecting well-known properties of glucocorticoids. This increase started 4 h after administration of the drug and lasted at least 20 h. A significant association between hyperglycemia and death has been demonstrated in septic patients.[22] Therefore, this observation could have important therapeutic consequences, given that hyperglycemia is a correctable abnormality that is common during critical illness and occurs even in patients without a previous history of diabetes.[23] Finally, the absence of any effects on plasma renin and aldosterone or on endogenous catecholamines underscores the fact that, under our experimental conditions, the treatments did not stimulate the renin–angiotensin–aldosterone and sympathetic nervous systems.

In addition to these biological effects, HC showed transient systemic hemodynamic effects whereas FC did not. There was an increase in cardiac output and a decrease of the same magnitude

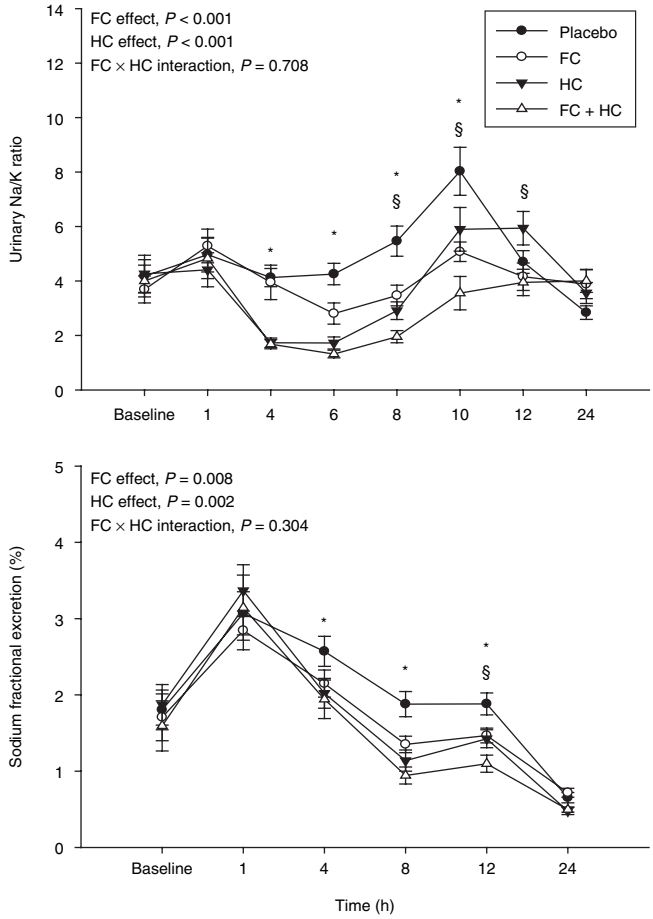

Figure 2 Changes in urinary sodium/potassium ratio and sodium excretion fraction with time. Data are mean ± SE of the mean. §FC effect compares the two periods during which subjects received FC (FC + HC placebo, FC + HC) with the two periods during which they did not (FC placebo + HC placebo, FC placebo + HC). *HC effect compares the two periods during which subjects received HC (FC placebo + HC, FC + HC) with the two periods during which they did not (FC placebo + HC placebo, FC + HC placebo). FC, fludrocortisone; HC, hydrocortisone.

in systemic vascular resistances, a moderate increase in heart rate, and no effect on stroke volume. Similar observations have previously been reported with respect to many glucocorticoids, such as dexamethasone, HC, prednisolone, methylprednisolone, and cortisol in normal subjects as well as in patients with various cardiopulmonary syndromes or shock.[24–26] In those studies, hemodynamic changes were observed between 90 and 240 min after high doses of steroids. Our data show that physiologic doses of HC have the same pharmacodynamic profile. The mechanisms responsible for these hemodynamic changes are not yet fully understood. It has been suggested that there may be a direct effect on systemic peripheral vasculature or a positive inotropic effect on the myocardium.[26] With regard to our results, the observed decrease in diastolic blood pressure argues in favor of a primary decrease in systemic vascular resistances. This hypothesis is in line with the observations that glucocorticoids are capable of downregulating β1-adrenergic receptors and upregulating β2-adrenergic receptors, resulting in a shift in the β1/β2 ratio.[27–29] It is therefore likely that the

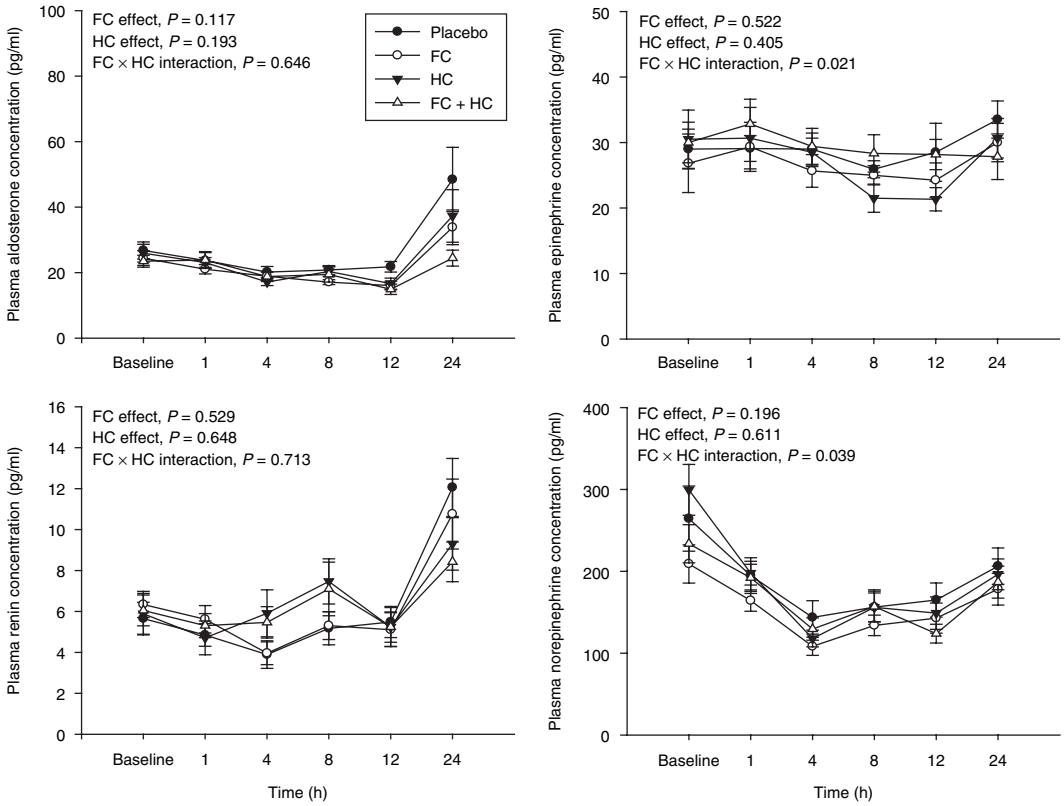

Figure 3 Changes in plasma aldosterone, renin, epinephrine, and norepinephrine concentrations with time. Data are mean ± SE of the mean. FC, fludrocortisone; HC, hydrocortisone.

increase in cardiac output was a response to the decrease in cardiac afterload resulting from the HC-induced upregulation of β2-adrenergic receptors. The increase in cardiac output could also have resulted from a direct inotropic effect of HC. Indeed, a slight but prolonged (3 h) inotropic effect has been observed with the use of prednisone in isolated cardiac tissue of cats.[30] Although the effect was not statistically significant in our study, stroke volume did register a slight increase at T4 after administration of HC (+7%), together with a small increase in heart rate (+4 beats/min). Therefore, we cannot rule out the possibility that HC has an inotropic effect. Specific echocardiographic (myocardial deformations) or invasive hemodynamic (left ventricular elastance) measurements of myocardial contractility, which were not performed in our study, would be necessary to confirm this hypothesis.

With respect to FC, previous studies have shown variable hemodynamic effects depending on physiological or pathological context and the dosage and duration of the treatment. In healthy volunteers, the administration of 1.5 mg/day (30 times the dose used in our study) for 5 days produced a rise in systolic blood pressure and a decrease in urinary sodium excretion, whereas 0.15 mg/day (three times the dose used in our study) for 5 days did not modify systolic blood pressure but had similar effects on urinary sodium excretion.[31] In patients with severe orthostatic hypotension, FC induced hypertension through a mechanism involving enhanced catecholamine responsiveness and increased systemic vascular resistances.[32] In this early study, the patients received doses that ranged from 0.3 to 1 mg/day (6–20 times the doses used in our study), and the hemodynamic effects on blood pressure and systemic vascular resistances were observed after 10 days of treatment. In our study in healthy volunteers, a single administration of 50 μg of FC did not modify blood pressure. However, it is noteworthy that (i) the profiles of effects on cardiac output and systemic vascular resistances observed after FC were consistently between those observed after placebo and those observed after HC, at 4 and 8 h, (ii) the decrease in systemic vascular resistances was perfectly compensated by the increase in cardiac output, and (iii) all these effects had disappeared by 12 h. These findings suggest that (i) FC is able to induce hemodynamic effects that are qualitatively similar to those of HC and (ii) the lack of statistical significance of these hemodynamic effects probably results from the low-dose and single-administration design used. Consideration of the results obtained in our study together with the results obtained in patients with severe orthostatic hypotension[32] suggests that FC may also exhibit effects similar to those of HC under conditions of hypotension and low systemic vascular resistance, both of which are key features of the hemodynamic profile of septic shock.

Our study has some limitations that require discussion. First, it was performed in healthy volunteers; even when we induced hypoaldosteronism to mimic adrenal insufficiency, the subjects continued to exhibit a normal hemodynamic profile in terms of blood pressure, cardiac output, and systemic vascular resistance.

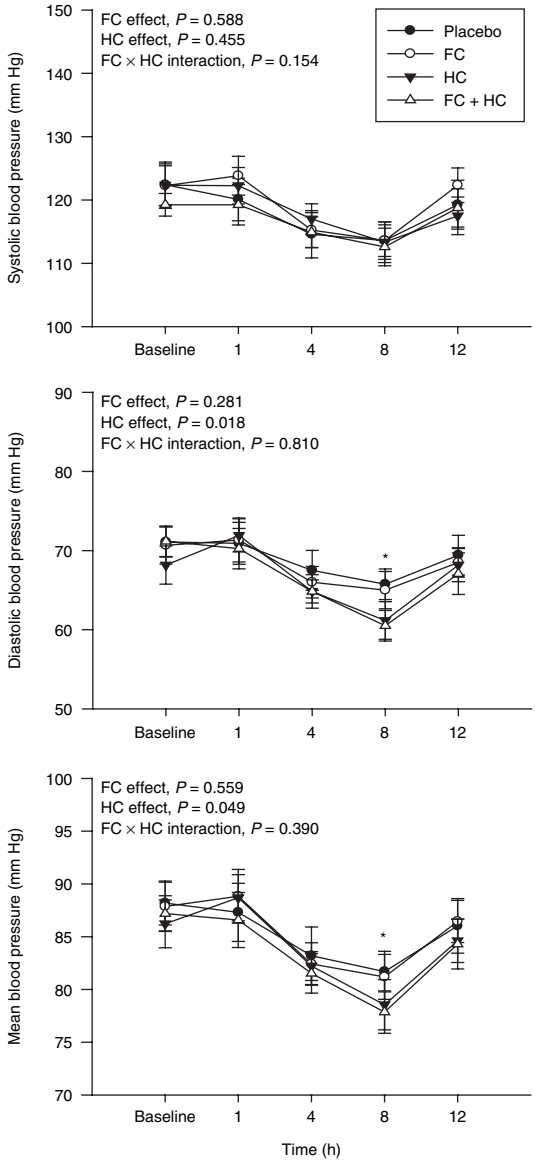

Figure 4 Changes in systolic, diastolic, and mean arterial blood pressures with time. Data are mean ± SE of the mean. *HC effect compares the two periods during which subjects received HC (FC placebo + HC, FC + HC) with the two periods during which they did not (FC placebo + HC placebo, FC + HC placebo). FC, fludrocortisone; HC, hydrocortisone.

Therefore, the hemodynamic effects of HC, and probably the lack of hemodynamic effects of FC as well, are not representative of what might happen during septic shock. Second, whereas treatment for septic shock consists of repeated administration of the two drugs, a study design with a single administration was chosen. Considering the delayed effects of FC on urinary sodium excretion, one could argue that the hemodynamic effects may have taken longer to establish and that the single administration design may have missed a delayed hemodynamic effect that might have taken place had administration been repeated. However, at the doses of FC used, this hypothesis is unlikely, both after single-dose administration (in view of the observed effects on cardiac output and systemic vascular resistances at 4, 8, and 12 h) and after repeated administration (in view of the results reported after administration of FC in healthy volunteers at a dosage of 0.15 mg/day for 5 days).[31] However, these results highlight the necessity for studying the effect of FC after repeated administrations and at larger doses in order to determine the optimal dose to be considered for use in septic shock.

In conclusion, we showed that, at the doses used in septic shock, a single administration of HC induced earlier and more pronounced mineralocorticoid effects than a single administration of FC did, and that HC induced systemic hemodynamic effects whereas FC did not. These results emphasize the necessity for undertaking new studies so as to better define the optimal dose of FC in septic shock.

METHODS

The study was approved by our regional committee for the protection of people in biomedical research (Comité de Protection des Personnes de Rennes Ouest V) on 5 March 2008 (08/08-667). All subjects gave written informed consent to participate.

Subjects. Twelve healthy male volunteers of age 24 ± 3 years and body mass index of 23.0 ± 1.9 kg/m^2 were included. Subjects were required to be nonsmokers and medication free. Before enrollment, they underwent clinical examination, 12-lead electrocardiogram, transthoracic echocardiography, drug screening of urine, and routine biological tests.

Protocol. This was a placebo-controlled, randomized, double-blind, crossover, four-period study performed according to a 2 × 2 factorial design. Each period was separated from the next by a washout interval of at least 14 days. Each subject received, in random order, FC placebo + HC placebo, FC + HC placebo, FC placebo + HC, or FC + HC. FC (50 µg) was administered orally, and HC (50 mg) was injected intravenously as a bolus. Drug doses and routes of administration were those used in patients with septic shock.[3] All experiments were conducted in a quiet, temperature-controlled (20 ± 2 °C) room.

All study periods were strictly identical. Subjects arrived at the clinical investigation unit of the INSERM 0203 Clinical Investigation Center of Rennes University Hospital at 6:45 AM after an overnight fast, and they were immediately placed in the supine position. At 7:30 AM, an indwelling catheter with a heparinized lock was inserted into a forearm vein of the left arm for blood sampling. At 7:45 AM, another catheter was inserted into a forearm vein of the right arm to allow intravenous infusion of 2,000 ml of saline over a period of 2 h. Such an acute volume expansion has previously been shown to induce significant decrease in plasma renin activity and aldosterone in healthy men.[33] At 8:00 AM, the subjects ate a standardized breakfast. When the saline infusion was completed, baseline (T0) biological and hemodynamic measurements were performed, and the relevant scheduled treatment was administered. The subjects remained in the hospital for 24-h periods for each of the treatments. Standardized lunch and dinner were given 4 and 9 h, respectively, after dosing. Blood samples were taken at 1, 4, 8, 12, and 24 h after T0 (T1, T4, T8, T12, and T24). Baseline aldosterone and renin–plasma concentrations were determined before the saline infusion in order to characterize the hypoaldosteronism that would be subsequently induced by volume expansion. Urine samples were collected 1, 4, 6, 8, 10, 12, and 24 h after T0. Hemodynamic measurements were repeated 1, 4, 8, and 12 h after T0 (T1, T4, T8, and T12).

The subjects were asked to have diets with normal levels of salt during the week before each treatment period. No alcoholic or caffeine-containing beverages were allowed from 48 h before each treatment period through the end of each period. During each treatment period, meals were served with 250 ml of water and 1 g of salt. Water supply was controlled and corresponded to half the urine volume.

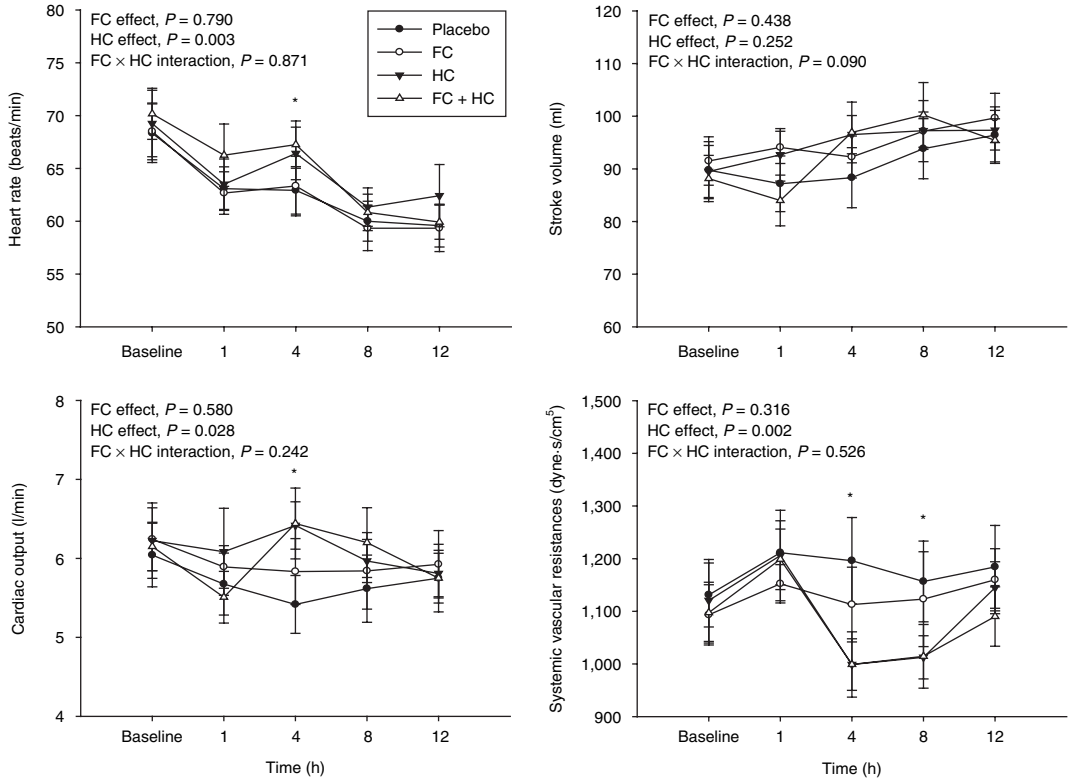

Figure 5 Changes in heart rate, stroke volume, cardiac output, and systemic vascular resistances with time. Data are mean ± SE of the mean. *HC effect compares the two periods during which subjects received HC (FC placebo + HC, FC + HC) with the two periods during which they did not (FC placebo + HC placebo, FC + HC placebo). FC, fludrocortisone; HC, hydrocortisone.

Biological variables. Plasma and urinary sodium, potassium, glucose, and creatinine levels were determined using standard biochemical techniques. Sodium fractional excretion was calculated as follows: (urine sodium × serum creatinine)/(serum sodium × urine creatinine).

Plasma aldosterone and renin concentrations were measured in subjects at rest after they had been lying in the supine position for at least 30 min. Blood samples were drawn into tubes containing no anticoagulant for aldosterone testing and EDTA for renin testing. The samples were immediately centrifuged at 1,000*g* for 10 min, and the plasma samples were stored at −80 °C until assay. Plasma aldosterone levels were measured by radioimmunoassay using a commercially available kit (Immunotech/Beckman Coulter, Villepinte, France). Plasma renin levels were measured by immunoradiometry, using the Renin III Generation kit (IBA/CisBio International Laboratories, Gif-sur-Yvette, France). Normal values were between 10 and 100 pg/ml for aldosterone and between 10 and 48 pg/ml for renin. The limits of detection were 6 pg/ml for aldosterone and 1 pg/ml for renin.

Plasma epinephrine and norepinephrine concentrations were measured by high-performance liquid chromatography with electrochemical detection.[34] Briefly, samples were drawn in lithium heparinate vacutainers and centrifuged for 10 min at 4 °C, and the plasma samples were stored at −80 °C until assay. Extraction was performed by the solid-phase extraction method, using a reagent kit (Chromsystems, Munich, Germany), before high-performance liquid chromatography analysis. Normal values were <100 pg/ml for epinephrine and <350 pg/ml for norepinephrine. The limits of detection were 10 pg/ml for both catecholamines.

Hemodynamic variables. Systemic systolic and diastolic blood pressures and heart rate were recorded noninvasively after 10 min of rest in the supine position, using a brachial sphygmomanometer (Dynamap ProCare; GE Healthcare, Freiburg, Germany). Mean blood pressure was calculated as 1/3 systolic blood pressure + 2/3 diastolic blood pressure. Pulse pressure was calculated as systolic blood pressure−diastolic blood pressure. Cardiac output, stroke volume, and systemic vascular resistances were measured noninvasively using an impedance cardiograph device (Physio Flow; Manatech Biomedical, Macheren, France), as previously described.[35] Aortic stiffness was estimated by means of carotid-ofemoral pulse wave velocity, using the foot-to-foot velocity method and a validated noninvasive device (SphygmoCor; AtCor Medical, West Ryde, Australia).[36] Briefly, pressure waveforms were sequentially measured at the carotid and femoral arteries by applanation tonometry, using a Millar piezoresistive pressure transducer (Millar SPT-301B; Millar Instruments, Houston, TX) coupled with the SphygmoCor device (AtCor Medical). Pulse wave velocity was then calculated as the distance between the two recoding sites divided by the time of travel of the pressure wave.

Statistical analysis. Statistical analysis was performed using SAS statistical software, version 9.1 (SAS Institute, Cary, NC). In text and tables, results are expressed as mean ± SD, whereas in figures they are presented as mean ± SE for clarity. Baseline variables measured at T0 in each of the four treatment periods were compared using a three-way analysis of variance (subject, period, and allocated treatment) taking into account the Latin square design. Thereafter, the changes over time of biological and hemodynamic variables were analyzed using a four-way analysis of covariance (subject, time, FC, and HC), adjusted on baseline values. All analyses were performed according to the 2 × 2 factorial design, yielding an FC effect and an HC effect and testing FC × HC interaction. The FC effect compared the two periods during which subjects received FC (FC + HC placebo, FC + HC) with the two periods during which they did not (FC placebo + HC placebo, FC placebo + HC). The HC effect compared the two periods during which subjects received HC (FC placebo + HC, FC + HC) with the two periods during which they did not (FC placebo + HC placebo, FC + HC placebo). FC × HC interaction assessed whether the effects of the two treatments were additive. If FC or HC

effects were significant, time-point comparisons were performed using the least-square means procedure according to the 2 × 2 factorial design. In time-point comparisons, the percentages of variation correspond to FC or HC effects, as described above. For all analyses, P values <0.05 were considered significant.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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