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Biochemical Evidence of the Functional Recovery and Regeneration of Adrenal Autotransplants in the Rat Spleen

Gonzalo Allende,[1] Roberto Chavira,[2] and Andrés Quintanar-Stephano[1]

[1]*Departamento de Fisiología y Farmacología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Av Universidad 940, Aguascalientes, Ags, 20100 México; and* [2]*Departamento de Biología de la Reproducción, Instituto Nacional de la Nutrición "Salvador Zubirán", Vasco de Quiroga 15, Delegación Tlalpan, México, DF, 14000 México*

Adrenal transplantation may restore adrenal function after bilateral adrenalectomy or when adrenal function is lost. Thus, animal experiments could provide useful information before clinical application of adrenal transplantation. Using an experimental model of autotransplantion of a complete adrenal gland in the spleen of adrenalectomized rats, several biochemical and hormonal parameters were studied to evaluate the function of transplanted adrenal tissue compared to control and adrenalectomized animals. Three weeks after surgery, the animals were sacrificed and plasma and tissue samples were obtained for biochemical studies. In the autotransplanted rats, plasma glucose, hepatic glycogen, plasma, and hepatic proteins, which were decreased in adrenalectomized rats, increased to values close to those of the control group; whereas muscle and thymus proteins, which were increased in adrenalectomized animals, decreased and reached normal levels. Corticosterone plasma levels in autotransplanted rats showed a 50% recovery compared to control animals, whereas plasma aldosterone concentrations were low, with similar values to those of the adrenalectomized group. These results provide evidence that the adrenal grafts secrete corticosterone in quantities enough to overcome hepatic inactivation. On the other hand, aldosterone plasma concentrations remain very low, plasma potassium levels are increased, and plasma sodium levels are decreased in animals with intrasplenic adrenal grafts, indicating that aldosterone production is insufficient to avoid hypoaldosteronism.

Key Words: Autotransplantation; adrenal; spleen; corticosterone; aldosterone.

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Author to whom all correspondence and reprint requests should be addressed: Gonzalo Allende, Depto. de Fisiología y Farmacología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Av. Universidad 940 C. P. 20100, Aguascalientes, Ags. México. E-mail: gdealle@correo.uaa.mx

Introduction

Adrenal autotransplantation in laboratory animals is a model commonly used to investigate adrenocortical regeneration *(1–4)* as well as adrenocortical zonation *(5–11)*. Moreover, transplantation of adrenal cortical tissue could represent a more physiologic approach to the treatment of adrenal insufficiency than lifelong corticosteroid replacement therapy *(12,13)*.

Succesful adrenal autotransplantation to the spleen of rats has long been used by several researchers *(1,14,15)*. Adrenals were also transplanted under the kidney capsule *(12,16)*, in the subcutaneous tissue *(4)*, in the musculus gracilis *(2,6)*, or between the dorsal skin and muscle *(17,18)*.

It is well known that adrenal autotransplants regenerate and partially restore their functions, since corticosterone blood levels but not those of aldosterone almost fully recover. It is also known that transplanted tissue does not achieve its normal morphological zonation *(2,4,5,9,17)*. However, the biochemical variations resulting from autotransplantation have received little attention. Since plasma ACTH levels are high following adrenal autotransplantation *(2,19,20)* and plasma renin levels are slightly increased *(5)*, the functional recovery can mainly be explained by the activation of the hypothalamus-pituitary-adrenal axis and the renin-angiotensin system. Adrenal grafts in the spleen deliver most of the secreted hormones via the splenic and portal veins to the liver. It is widely known that corticosteroid hormones are inactivated by enzymatic conjugation in the liver; at the same time, glucocorticoids secreted by the adrenal graft affect intrahepatic carbohydrate, protein, and lipid metabolism. Thus, in rats with adrenal autografts in the spleen, the secreted corticosterone and aldosterone could be metabolized by the liver before they reach the peripheral circulation, at least during the first few weeks after transplantation, inducing extra-hepatic adrenal insufficiency. It could also be assumed that the feedback mechanisms exerted by the hypothalamus-pituitary-adrenal axis and the renin-angiotensin system would stimulate the adrenal graft to secrete corticosteroids, in order to overcome hepatic inactivation and reestablish the normal biochemical conditions. Therefore, the aim of the present work was to evaluate the functional activity of the splenic adrenal transplants

Table 1
Effects of Adrenal Autotransplantation in the Rat Spleen on Biochemical and Hormonal Parameters[a]

	(1) CONT	(2) SHAM	(3) ADX	(4) ADX+SP
Plasma glucose (mg/dL)	93.5 ± 4.1	75.4 ± 7.3 a vs 1	49.2 ± 6.4 d vs 1 b vs 2	91.0 ± 3.9 a vs 1,2 d vs 3
Hepatic glycogen (mg/100 mg)	2.58 ± 0.24	2.34 ± 0.25 a vs 1	0.90 ± 0.15 d vs 1,2	2.48 ± 0.25 a vs 1,2 d vs 3
Total plasma proteins (mg/mL)	77.9 ± 2.6	81.7 ± 3.8 a vs 1	54.7 ± 2.8 d vs 1,2	75.1 ± 1.8 a vs 1,2 d vs 3
Total hepatic proteins (mg/g)	372.2 ± 17.8	370.4 ± 24.1 a vs 1	245.0 ± 24.4 c vs 1,2	354.5 ± 31.3 a vs 1,2 b vs 3
Diaphragma proteins (mg/g)	164.2 ± 7.6	158.4 ± 8.8 a vs 1	222.3 ± 5.8 d vs 1,2	163.9 ± 3.5 a vs 1,2 d vs 3
Thymus proteins (mg/g)	94.4 ± 6.7	96.7 ± 3.9 a vs 1	142.1 ± 6.6 d vs 1,2	94.4 ± 6.4 a vs 1,2 d vs 3
Plasma triglycerides (mg/dL)	82.6 ± 7.8	80.7 ± 7.0 a vs 1	42.3 ± 4.7 b vs 1,2	52.8 ± 13.2 a vs 1,2,3
Plasma cholesterol (mg/dL)	61.3 ± 1.5	65.1 ± 4.5 a vs 1	52.8 ± 4.6 a vs 1,2	65.3 ± 4.5 a vs 1,2,3
Plasma potassium (mEq/L)	3.84 ± 0.13	3.72 ± 0.29 a vs 1	5.55 ± 0.52 b vs 1,2	6.32 ± 0.90 c vs 1,2 a vs 3
Plasma sodium (mEq/L)	130.4 ± 1.9	111.6 ± 15.8 a vs 1	92.0 ± 4.4 b vs 1 a vs 2	92.8 ± 4.6 b vs 1 a vs 2,3
Plasma corticosterone (ng/mL)	123.6 ± 8.7	132.3 ± 11.0 a vs 1	23.4 ± 9.5 d vs 1,2	61.4 ± 4.2 d vs 1,2 b vs 3
Plasma aldosterone (pg/mL)	46.4 ± 6.7	47.8 ± 6.8 a vs 1	15.0 ± 7.1 b vs 1 c vs 2	16.0 ± 4.5 c vs 1,2 a vs 3

[a]The data presented are the means ± SEM (n = 7–10). Statistical comparison of the data (p <): a = not significant ($p > 0.05$), b = $p < 0.05$, c = $p < 0.01$, and d = $p < 0.001$.

in adrenalectomized rats, by measuring several biochemical parameters and comparing them with those found in control and adrenalectomized animals.

Results

In comparison with the control group (CONT), the adrenalectomized rats (ADX) showed a significant decrease of plasma glucose (53%), hepatic glycogen (35%), total plasma proteins (70%), total hepatic proteins (66%), plasma triglycerides (51%), cholesterol (86%), and plasma sodium (71%) (Table 1). The decrease in these parameters coincided with the reduction of plasma corticosterone (19%) and aldosterone (32%) concentrations (Figs. 1A, 1B, and Table 1). The ADX animals had a significant elevation in diaphragmatic muscle (35%) and thymus (50%) total proteins, and in plasma potassium (44%) (Table 1). In the adrenalectomized rats with intrasplenic adrenal autotransplants (ADX+SP), plasma glucose (97%), hepatic glycogen (96%), plasma (96%) and hepatic (95%) proteins, and cholesterol (106%), reached values close to those of the control group (Table 1). As the percentages in parentheses indicate, muscle (99%) and thymus (100%) protein values returned to normal (Table 1). However, in the ADX+SP animals, plasma potassium increased 65% and plasma sodium decreased 29% compared with the CONT group, showing no reestablishment toward control values (Table 1). No total recovery of plasma triglycerides was evident (64%) in ADX+SP group (Table 1).

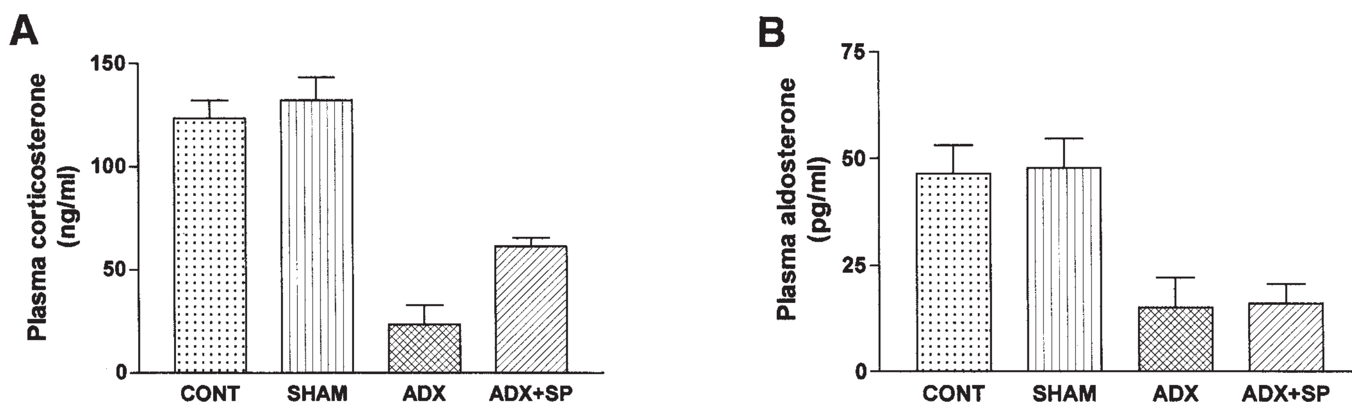

Fig. 1. Effects of adrenal autotransplantation in the rat spleen on (**A**) plasma corticosterone (ng/mL plasma) and (**B**) plasma aldosterone (pg/ml plasma). Each bar represents the mean ± SEM of values from 7 to 10 animals and the statistical comparisons between groups are shown in Table 1.

Table 2
Body Weights (g) in Control, Sham-Operated, ADX, and ADX+SP Rats at Different Days of the Experimental Period[a]

Day	(1) CONT	(2) SHAM	(3) ADX	(4) ADX+SP
0 (before surgery)	259.8 ± 6.5	271.5 ± 5.2	255.9 ± 4.5	263.2 ± 6.3
7	261.2 ± 6.0	282.3 ± 7.2	261.8 ± 4.1	262.3 ± 9.2
14	282.6 ± 5.0	288.3 ± 7.3	263.4 ± 4.3	268.5 ± 10.5
21 (before sacrifice)	273.6 ± 5.3	281.5 ± 6.4	266.4 ± 5.6	266.8 ± 7.8

[a]Results are means ± SEM (n = 10).

The changes of the metabolic parameters observed in the autotransplanted animals were in accordance with the corticosterone and aldosterone plasma levels. The plasma corticosterone showed a 50% recovery in relation to the CONT rats (Fig. 1A and Table 1), accounting for the reestablishment of plasma glucose, hepatic glycogen, and total proteins measured in plasma, liver, diaphragmatic muscle, and thymus (Table 1). In contrast, aldosterone concentrations in the ADX+SP group showed similar values to those of the ADX group (Fig. 1B and Table 1), providing an explanation for the increase of plasma potassium and the decrease of plasma sodium compared with the CONT group (Table 1).

No important differences were found among the average weights of each group, measured before surgery and at d 7, 14, and 21 after surgery. A slight and comparable weight increment was detected in all groups (Table 2). Daily water intake and urine excretion showed no statistically significant differences among the experimental groups, except for the adrenalectomized animals, in which water consumption and urine excretion were higher than in the other groups, particularly in the first week after surgery (Tables 3 and 4). This result can be reasonably explained by the complete absence of mineralocorticoid secretion in the ADX rats.

The histological studies showed that, 21 d after transplantation, adrenal grafts in the spleen were easily identified by their yellow color. Using light microscopy the grafts showed different elements: (1) a thick but incomplete capsule of fibroblasts and collagen fibers surrounding the graft (Fig. 2A); (2) spherical or ovoid nodules of fasciculata-like cells under the capsule and disseminated within the spleen (Fig. 2A). As the zona fasciculata in normal adrenals, nodules in adrenal transplants were composed of polyhedral cells with central nucleus. Cells were arranged in columns one or two layers thick, placed radially (not shown). With hematoxylin–eosin (H-E) staining, the cytoplasm is acidophilic with clear vacuoles left by extraction of lipids (Fig. 2B). The size of the nodules fluctuated between 60 and 700 µm in diameter. (3) The largest components of the grafts were big cells with cytoplasmic vacuoles and picnotic nuclei (Fig. 2B); (4) foci of chronic inflammatory reaction composed of a mixture of macrophages, lymphocytes, and fibroblasts (not shown); (5) cell remnants resembling the medulla of the adrenal gland. In areas without capsule (sites where the adrenals were cut by the surgical procedure), fibroblasts, connective tissue fibers, and degenerating adrenocortical-like cells were mixed with splenic vessels that seemed to invade the graft (Fig. 2B). Zonation was not apparent, since

Table 3
Water Intake (mL/24 h per 100 g body weight)
in Control, Sham-Operated, ADX, and ADX+SP Rats[a]

Week	(1) CONT	(2) SHAM	(3) ADX	(4) ADX+SP
First	24.9 ± 2.4	30.9 ± 3.1 a vs 1	136.3 ± 3.6 b vs 1,2	23.3 ± 1.9 a vs 1,2 b vs 3
Second	27.9 ± 2.7	24.5 ± 1.8 a vs 1	45.2 ± 2.9 b vs 1,2	25.6 ± 2.4 a vs 1,2 b vs 3
Third	25.9 ± 1.2	20.9 ± 1.6 a vs 1	41.5 ± 1.1 b vs 1,2	25.0 ± 1.7 a vs 1,2 b vs 3

[a] Values are means ± SEM ($n = 6$–8) of daily water consumption at each week of the experimental period. Statistical comparison of the data ($p <$): a = not significant ($p > 0.05$) and b = $p < 0.001$.

Table 4
Urine Excretion (mL/24 h per 100 g body weight)
in Control, Sham-Operated, ADX, and ADX+SP rats[a]

Week	(1) CONT	(2) SHAM	(3) ADX	(4) ADX+SP
First	10.2 ± 0.6	13.6 ± 1.2 a vs 1	79.2 ± 8.4 b vs 1,2	12.5 ± 0.8 a vs 1,2 b vs 3
Second	7.6 ± 0.6	8.2 ± 0.5 a vs 1	15.1 ± 0.8 b vs 1,2	9.8 ± 0.9 a vs 1,2 b vs 3
Third	8.6 ± 0.5	8.3 ± 0.4 a vs 1	16.3 ± 0.7 b vs 1,2	10.8 ± 1.0 a vs 1,2 b vs 3

[a] Data are means ± SEM ($n = 6$–8) of daily urine volumes at each week of the experimental period. Statistical comparison of the data ($p <$): a = not significant ($p > 0.05$) and b = $p < 0.001$.

glomerulosa and fasciculata layers could not be identified in the grafts.

Discussion

Adrenal transplantation may be a treatment option after bilateral adrenalectomy or complete loss of adrenal function. Thus, animal experiments could provide useful information before clinical application of adrenal transplantation. Our present findings dealing with complete autotransplanted adrenals into the rat spleen, and corticosterone and aldosterone secretion by the autografts, are in agreement with those reported by Belloni et al. *(5)*. These authors transplanted adrenal fragments to the musculus gracilis. As suggested by Belloni et al. *(1)*, we also suppose that in our experimental model, the temporary loss of the negative feedback mechanism (which initially enhances ACTH release in response to bilateral adrenalectomy) may be responsible for the subsequent regeneration, since the venous blood of the spleen drains via the portal vein into the liver, where corticosteroids are metabolized. It is known that the transplanted adrenals do not regenerate in the spleen of hypophysectomized rats *(14)*.

Our results show that animals with intrasplenic adrenal grafts display a partial recovery (50%) of plasma corticosterone levels, but not of aldosterone levels (Table 1). These results are consistent with those reported by Vendeira et al. *(18)*, but are at a slight variance with the findings reported in an earlier publication by the same authors *(17)* and by Belloni et al. *(2,5)*, who described an almost complete normalization of blood corticosterone levels 4 mo after transplantation. The reason for this difference could be the shorter period between surgery and sacrifice (3 wk) in our study, which only would allow an incomplete regeneration of the

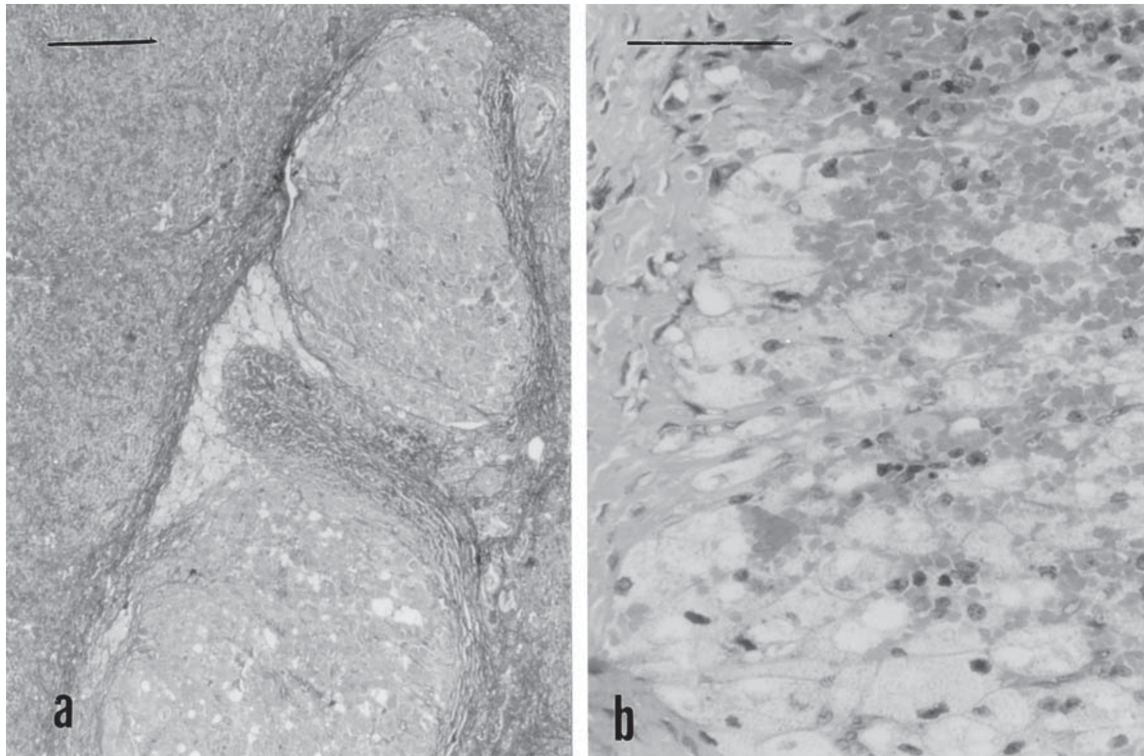

Fig. 2. Light micrographs of (**A**) nodules of regenerating adrenocortical cells, 21 d after autotransplantation in the spleen. The graft is surrounded by a thick capsule of collagen fibers and two nodules with fasciculata-like adrenocortical cells. Note the atrophied adrenocortical-like cells between the nodules (clear cells). Magnification: ×40. Bar = 150 µm. Stain: Mallory–Heidenhain. (**B**) Same graft as **A** but from another area. The graft contains adrenocortical cells with signs of atrophy and large cells with cytoplasmic granules, empty vacuoles of different size and picnotic nuclei. Note the fibrous capsule at the left, and splenic vessels invade the graft at the right. Magnification: ×200. Bar = 50 µm. Stain: hematoxylin–eosin (H-E).

transplanted tissue to normalize corticosterone secretion. Saxe and Connors *(21)* also found that overall corticosterone response was smaller in the transplanted group compared with the sham-operated group; they suggested that this may be due to differences in adrenal mass, responsiveness of transplanted tissue, or inaccessibility of transplanted tissue to trophic hormones and nutrients. Vendeira et al. *(18)* noted that serum corticosterone was 50% lower in autotransplanted rats than in sham-operated animals, and this might be due to a relatively low adrenocortical mass present in the transplanted animals *(2,19,20)*. However, the partial recovery of plasma corticosterone levels obtained in our experiments was sufficient to cause normalization of the biochemical parameters regulated by this hormone (plasma glucose, hepatic glycogen, and plasma, hepatic, muscle, and thymus proteins).

Conversely, there was an impairment of aldosterone secretion by the transplanted adrenal, which was reflected in the failure to recover potassium and sodium plasma concentrations in the autotransplanted animals (Table 1). These findings are in complete agreement with those of previous studies *(2,5,18)*. The reduction of aldosterone secretion by the transplanted adrenal may be explained by several possibilities. Our technique involves the transplantation of the whole adrenal into the rat spleen, probably producing a larger necrosis of the transplanted tissue than other techniques, e.g., the one involving the implantation of small capsular fragments. This latter technique most likely avoids the massive necrosis that affects transplanted adrenal halves or quarters during the first postoperative week *(1,22)*, since it facilitates vascularization and nutrition of parenchymal cells, thus enhancing their proliferation rate *(2)*. Large necrosis in the transplant would delay complete regeneration of adrenocortical tissue and, consequently, restoration of normal blood corticosteroids levels. The site for implantation could also be important. Although the splenic parenchyma, with its extensive blood flow, may represent a favorable environment for adrenal regeneration, venous blood from the spleen drains via the portal vein into the liver, where adrenocortical hormones are rapidly metabolized delaying normalization of their blood levels.

The histological studies carried out by other researchers provided valuable information *(2,5,17)*. Adrenal regeneration in the autotransplant occurs at the graft periphery and proceeds from subcapsular glomerulosa cells. Initially, there is an extensive necrosis of glandular cells of the zonae

fasciculata, reticularis, and part of the glomerulosa *(17)*. The cells of the zonae fasciculata, reticularis, and medulla undergo necrosis and are subsequently reabsorbed. The capsule forms a sphere, within which the glomerulosa cells proliferate and generate a new functional zona fasciculata. Thus, the gland regenerates from glomerulosa cells. There are also large subcapsular glomerulosa cells filled with lipid droplets. The medulla is, however, absent *(17)*. Regenerated adrenocortical nodules are surrounded by a thin connective tissue capsule, from which few trabecula detach and dip into the parenchyma to form connective tissue septa. The nodules contain no chromaffin cells and do not display the classical zonal arrangement of the adrenal cortex. Our histological findings were very similar to those described above (*see* Results). Most of the parenchymal cells in the regenerating adrenal transplant, closely resemble those of the zonae fasciculata and reticularis in control animals *(2)*. This latter observation could account for the low plasma aldosterone levels measured in the transplanted animals, since this hormone is produced by glomerulosa cells that are scarce in the regenerating tissue. The smaller number of glomerulosa cells in relation to corticosterone-secreting fasciculata cells is not only evident in regenerated transplanted tissue, but also in the normal adrenal cortex. In fact, the physiological aldosterone plasma levels are within the range of picograms per milliliter, whereas corticosterone levels are usually expressed in nanograms per milliliter. Thus, it is reasonable to assume that corticosterone secretion by the more numerous fasciculata cells in regenerating tissue could overcome hepatic inactivation easier than aldosterone secretion by the less numerous glomerulosa cells.

In view of the increase of plasma ACTH levels in the autotransplanted rats after bilateral adrenalectomy *(2,19, 20)*, it is conceivable that ACTH not only induces conversion of glomerulosa cells to fasciculata cells, but also accelerates the centripetal movement of cells from the capsule to the medullary border in the growing rat adrenals *(23,24)*. This could also delay the regeneration of zona glomerulosa cells and, consequently, retard and/or prevent the normalization of aldosterone plasma levels, since these cells would be mainly involved in the regeneration of zona fasciculata cells and zonation of newly formed adrenocortical tissue. It was also suggested that the absence of splanchnic innervation and the loss of adrenal medullary hormones may be responsible for an incomplete stimulation under basal conditions and a decrease of mineralocorticoid production *(5)*.

In summary, two major conclusions can be drawn from the present study: first, the restoration of plasma glucose, hepatic glycogen, and plasma, hepatic, muscle, and thymus proteins in the autotransplanted animals indicates that the adrenal graft secretes corticosterone in quantities enough to overcome hepatic inactivation, to reach various tissues and carry out their metabolic functions; and second, aldosterone production in the splenic graft is insufficient to reestablish potassium and sodium plasma levels. Further experiments assaying plasma corticosteroid concentrations in the portal venous blood and systemic circulation will be useful to evaluate the influence of hepatic inactivation on corticosterone and aldosterone secretion by the adrenal grafts implanted to the spleen.

Materials and Methods

Twelve to fourteen week old male Wistar rats weighing 230–290 g were obtained from the colony of the University of Aguascalientes and divided into the following four groups ($n = 7–10$): *(1)* Control (CONT), *(2)* Sham-operated (SHAM), *(3)* Adrenalectomized (ADX), and *(4)* Adrenalectomized with autotransplant of adrenal gland in the spleen (ADX+SP). Autotransplantation involved bilateral adrenalectomy performed through a dorsal incision under anesthesia with sodium pentobarbital (30 mg/kg ip); the adrenals were placed in a 0.9% NaCl sterile solution. The right gland was discarded. The left gland was cleaned of all surrounding fat and connective tissue. Two cuts were made to the gland in such a way that the gland could be stretched. With a thin needle (Ob 228/4) and a silk thread (6-0), a stitch was made at the end of the excised gland in order to hold it. A wide and straight needle (A 202/2) threaded with the silk thread holding the adrenal was inserted into the spleen parenchyma. The thread was then cut and separated from the adrenal gland. No more than 10 minutes elapsed between adrenalectomy and the completion of the transplant. All animals were fed with standard Purina chow. The adrenalectomized rats were given 0.9% NaCl drinking solution during the entire experimental period (3 wk) before sacrifice. In the first week after surgery, the animals with the splenic adrenal transplants received a drinking solution of 0.9% NaCl. In the second week the NaCl concentration was reduced to 0.5%, and, in the third week, they were mantained with tap water. The control and sham-operated rats were supplied with tap water until the sacrifice. The rats were housed under normal laboratory conditions with regular diurnal light/dark alterations (12 h light/12 h darkness cycles). Animals were weighed at d 0, 7, 14, and 21 of the experimental period to determine the average weights in each group. Urine volumes were measured using metabolic cages. Daily water consumption was also quantified. Three weeks after surgery, rats were sacrificed by decapitation under light ether anesthesia and trunk blood was collected for biochemical measurements. Animals were handled gently by the same operator to minimize stress. The plasma was separated by centrifugation and stored at –20ºC until required for assays. The animals were autopsied immediately after the sacrifice to obtain the liver, diaphragmatic muscle (sternal and costal parts), and thymus, which were stored at –20ºC until total proteins and hepatic glycogen measurements were performed. Spleens with the adrenal graft were fixed in 10% neutral formaline; then, they were dehydrated, embedded in paraffin, and transversally sectioned at 5–6 μm for histol-

ogy. Sections were stained with hematoxylin–eosin (H-E) or the Mallory–Heidenhain's staining. Total proteins of the various tissues and plasma were determined by the method of Bradford *(25)*. Hepatic glycogen was measured with the procedure described by Murat and Serfaty *(26)*. Plasma glucose was determined using the o-toluidine method. Potassium (K^+) and sodium (Na^+) concentrations were measured in a Corning Flame Photometer 410 (UK). Plasma triglycerides and cholesterol were determined using commercial kits purchased from Hycel (Mexico) and Randox (UK), respectively. Plasma corticosterone and aldosterone were determined using Coat-A-Count rat corticosterone and rat aldosterone radioimmunoassay kits purchased from Diagnostic Products Corporation (Los Angeles, CA, USA; intra- and interassay variations for corticosterone were 12.7% and 18.4%, respectively, and for aldosterone 13.2% and 19.1%). All measurements were carried out in triplicate and repeated at least twice. Results are expressed as the mean ± SEM. The differences between means were compared with an ordinary ANOVA and Tukey multiple comparison tests. A p value of less than 0.05 was considered statistically significant. All other commercial reagents used in the experiments were purchased from Sigma Chemical Co. (St Louis, MO, USA).

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