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Effect of gonadectomy on cyclosporine pharmacokinetics in male and female rats

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The present paper reports about the effect of gonadectomy on cyclosporine (CyA) pharmacokinetics in rats. The oral administration of CyA (10 mg/kg b.w.) to male rats caused two-fold higher drug blood levels than those reached by females at 24 h after the last dose (334.10 ± 126.70 vs. 161.49 ± 53.39 ng/ml, $p < 0.05$). These levels increased by about 25% in orchiectomized male rats (419.47 ± 132.63 ng/ml) but they returned to control values after testosterone treatment (330.99 ± 130.80 ng/ml). On the other hand, CyA blood levels (90.66 ± 22.25 ng/ml) decreased after ovariectomy, even more in the case of gonadectomized female rats receiving estradiol replacement (67.83 ± 24.15 ng/ml). With regards to drug distribution, the concentrations of CyA in the liver, the kidneys and the spleen at 24 h after the last dose were about 8, 5 and 6-fold higher than blood levels, respectively, regardless of animal gender. These partition coefficients were increased to 11, 7 and 9-fold by male castration suggesting a more extensive drug distribution. Contrariwise, drug tissue levels in ovariectomized rats decreased. The changes of drug blood and tissue levels among groups were not associated to the variations of metabolite concentrations in the liver or blood. Therefore, gonadectomy exerts a complex effect on CyA pharmacokinetics in rats and makes complementary studies necessary to clarify how differences in sexual hormone secretion alter CyA disposition.

1. Introduction

In recent years, data on human kidney and heart transplantation have shown a sex-associated difference in the survival time of the graft with females rejecting organ grafts earlier than males [1–3]. Likewise, experimental transplantation of heart and skin grafts in rats have also demonstrated the same rejection pattern [2, 4, 5]. However, gonadectomy and hormonal therapy reversed this rejection pattern in rats [5]. These data suggest a strong influence of gender on the immune response modulation but a potential effect on the pharmacokinetics of immunosuppressants cannot be neglected. Several preclinical and clinical investigations have shown a strong influence of gender on the pharmacokinetics of the potent immunosuppressant cyclosporine (CyA). For humans, the results from several papers are contradictory; some suggest that women clear the drug slower than men [6] whereas more recent research [7–10] shows evidence for a faster clearance in women. In vitro studies [11–13] have also shown conflicting results in rats; however, *in vivo* studies [2, 11, 14–17], involving either acute or chronic CyA dosing, demonstrated higher whole blood CyA levels in males than female rats. CyA undergoes extensive metabolism through the predominant CYP450 3A gene family. In rats, the expression of several CYP450 as well as phase-two isoenzymes is sex-dependent [18, 19]. In fact, CYP450 3A2, the major metabolizing enzyme for CyA, is constitutively present in mature male rats but absent in females. More recent papers [11, 13] suggest that isoenzymes other than 3A2 but 3A related should account for a significant amount of CyA metabolism in rats. In this sense, new 3A related female specific isoenzymes have been identified [20, 21]; although none of them have yet been proven to metabolize CyA, some evidence suggest that they might be involved. Similarly, the formation of sulfates or glucuronides is known for CyA or its metabolites in humans, albeit it has not yet been proven in the rat.

The sex-dependent expression of these rat hepatic enzymes is regulated mainly by the sex-specific pattern of growth hormone secretion and is subject to androgen imprinting [22]. Furthermore, gonadectomy drastically downregulates their expression, which can be restored by hormonal therapy [21, 23, 24]. So then, it is possible that the differences found in CyA disposition among males and females may be partly responsible for the sex-associated rejection pattern previously observed in rats. Due to gonadectomy reverses the early graft rejection experimented by female rats and it alters the expression of several isoenzymes potentially involved in CyA biotransformation, the present study has been focused at identifying the effect of gonadectomy and subsequent hormonal restoration therapy on CyA pharmacokinetics in male and female rats, in order to gain more insight on the gender dependency of CyA pharmacokinetics previously observed in rats. It is generally accepted that whole blood trough levels within 100 and 300 ng/ml provide adequate immunosuppression and no toxicity [25]. Consequently, the data described herein refer to blood and tissue CyA levels obtained at 24 h post-dosing from orally treated rats.

2. Investigations and results

The analysis of blood and tissue samples from control groups receiving the vehicles of CyA and hormones showed that the drug concentrations were all below the limit of quantification suggesting an acceptable specificity of immunoassay methods. Figure 1 shows the blood trough levels achieved in those groups receiving the drug. As shown, the average CyA blood trough concentrations achieved in control males were about two-fold higher ($p < 0.05$) (334.10 ± 126.70 ng/ml) than in females (161.49 ± 53.39 ng/ml). Orchiectomy (ORCHx) induced a slight increase (~25%) of these values (419.47 ± 132.63 ng/ml) but the administration of testosterone (TT) reversed the drug blood levels (330.99 ± 130.80 ng/ml) to those found in sham-operated male rats; notwithstanding, the large variability associated to CyA pharmacokinetics caused the differences were not statistically significant. The results suggested a rather different behavior for females. In fact, CyA blood levels significantly decreased

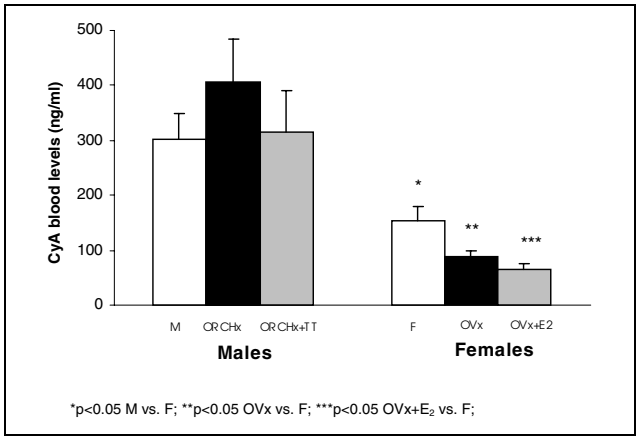

Fig. 1: CyA concentrations (mean ± s.e.m.) in blood of Wistar rats 24 h after the last dose. Key: sham operation of males (M) and females (F); gonadectomized males (ORCHx) and females (OVx) and gonadectomized plus testosterone treatment (ORCHx + TT) or estradiol treatment (OVx + E2)

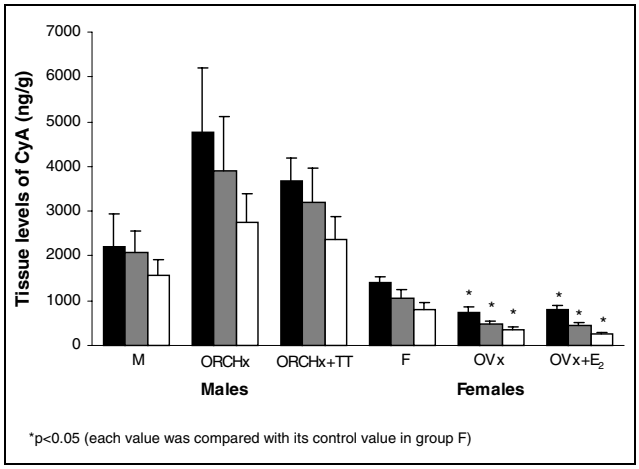

Fig. 2: Hepatic (black bars), splenic (gray bars) and renal (white bars) levels of CyA (mean ± s.e.m.) 24 h post-treatment. Key: sham operation of males (M) and females (F); gonadectomized males (ORCHx) and females (OVx) and gonadectomized plus testosterone treatment (ORCHx + TT) or estradiol treatment (OVx + E2)

($p < 0.05$) in ovariectomized (OVx) rats (90.66 ± 22.25 ng/ml) as compared to control females being this effect more pronounced when estradiol and CyA were coadministered to gonadectomized females. Indeed, CyA blood levels in OVx + estradiol (E2) rats (67.83 ± 24.15 ng/ml) were significantly ($p < 0.05$) reduced to more than one half of control values (Fig. 1).

The same differences among control males and females were observed when drug tissue contents were considered (Fig. 2). Nonetheless, the distribution of CyA into the liver, the spleen and the kidneys at 24 h post-treatment showed no gender dependency as shown by the corresponding tissue to blood partition coefficients (K_p); drug levels in the liver, spleen and kidneys were about 8-fold, 6-fold and 5-fold higher than blood levels, respectively (Table 1). Male castration caused an increase of drug tissue distribution as indicated by the two-fold higher CyA tissue levels reached in ORCHx rats than control group at 24 h post-treatment (Fig. 2). However, as occurred with drug blood levels, the large variability of data showed non significant differences from a statistical standpoint. The administration of TT did not modify the tissue contents of CyA in ORCHx rats (Fig. 2). Despite the concentrations of CyA in the different tissues showed no statistical significant differences among groups, the K_p values for the liver, kidneys and spleen of ORCHx rats, either TT supplemented or not, indicated 11-fold, 7-fold and 9-fold higher concentrations than blood trough levels, respectively. Nonetheless, these increments only were statistically significant for the kidneys and the spleen ($p < 0.05$).

In females, the drug contents found in the liver, the spleen and the kidneys of OVx rats fell all below the corresponding control values ($p < 0.05$), however, the K_p values suggested that only the distribution of CyA into the kidney and spleen was decreased (Table 1). The treatment of OVx rats with E2 did not induce significant changes of tissue concentrations as compared to gonadectomized but non-supplemented females and the initial equilibrium between spleen and blood levels was restored. As opposed, E2 administration did not vary the partition of CyA between the kidneys and blood after female gonadectomy.

The indirect measurement of CyA metabolites in blood evidenced very low levels of circulating metabolites at 24 h post-treatment. In fact, the average blood levels in control female rats were all below 21 ng/ml while it amounted to 64.31 ± 39.55 ng/ml in sham-operated males (Fig. 3). Likewise, the liver of control male rats accumulated more extractable metabolites than control females. Globally, they represented much higher levels than those achieved in blood and gender did not affect the percentage of total CyA derivatives corresponding to metabolites in the liver and blood (liver: $53.44 \pm 11.11\%$ in males vs.

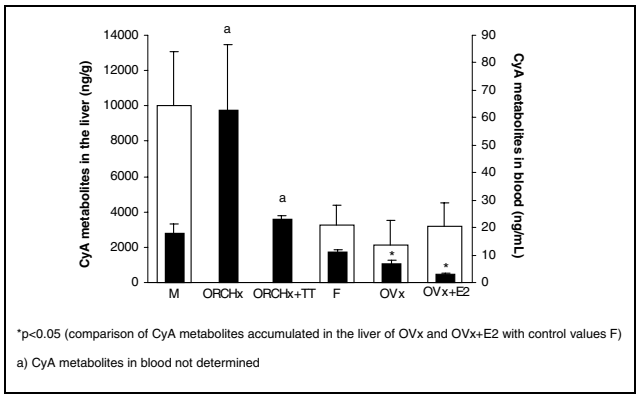

Fig. 3: Amounts of circulating metabolites (mean ± s.e.m.) in blood (empty bars) or accumulated in the liver (shaded bars) of Wistar rats 24 h after the last dose. Key: sham operation of males (M) and females (F); gonadectomized males (ORCHx) and females (OVx) and gonadectomized plus testosterone treatment (ORCHx + TT) or estradiol treatment (OVx + E2)

Table: Tissue to blood partition coefficients (K_p) (mean ± s.d.) of CyA in male and female rats 24 h after treatment

	M	ORCHx	ORCHx + TT	F	OVx	OVx + E_2
Liver/blood	7.30 ± 3.19	11.73 ± 1.88	11.61 ± 2.11	9.18 ± 4.43	8.17 ± 3.62	12.49 ± 2.23
Kidney/blood	5.25 ± 0.94	6.74 ± 0.53#	7.57 ± 0.68#	5.30 ± 0.59	4.14 ± 0.54*	3.98 ± 0.53*
Spleen/blood	5.98 ± 1.76	8.91 ± 1.98	9.62 ± 0.62#	6.52 ± 0.42	5.14 ± 0.30*	6.58 ± 0.59

Significant difference ($p < 0.05$) when compared to the corresponding control values (M)
* Significant difference ($p < 0.05$) when compared to the corresponding control values (F)

54.52 ± 5.95% in females; blood: 14.83 ± 8.88% in males vs. 11.62 ± 4.37% in females). On one hand, orchiectomy induced a strong accumulation of CyA metabolites in the liver but this resulted only in a slight and non-significant variation for the percentage of total CyA compounds represented by metabolites (62.63 ± 11.97%). Furthermore, the differences were abolished by TT supplementation, being the metabolites 49.60 ± 4.60% of total CyA compounds determined. On the other hand, the average amount of CyA metabolites in the liver of gonadectomized females decreased to 62% ($p < 0.05$) of control values. This reduction was proportional to the variation of parent drug levels in the liver since the equilibrium between metabolites and total CyA compounds remained unchanged (57.05 ± 13.46%). Contrariwise, gonadectomy and subsequent administration of E2 decreased the hepatic abundance of CyA metabolites to 27% ($p < 0.05$) of control values and these amounted only to 35.18 ± 7.68% of total CyA compounds determined in the liver ($p < 0.05$).

3. Discussion

Present results confirm the dependency of CyA pharmacokinetics on gender under different dosing schedules [2, 14–16]. This difference may be relevant since the rejection pattern observed in experimental transplantation [1, 2, 4, 5] could be partly due to the lower blood and tissue CyA levels in female rats. It is well known that the isoenzymes involved in CyA biotransformation belong to the 3A subfamily [12] and are subjected to sexual imprinting [18]. Furthermore, their expression is drastically reduced by gonadectomy but it can be restored by hormonal replacement therapy [21, 23]. In this study, only slight variations of CyA blood concentrations were detected among the groups of males. Although, they were qualitatively in agreement with the previously reported changes of CYP450 3A2 expression induced by castration and hormonal treatment, more significant modifications of blood trough levels should be expected. In females, the higher blood trough levels found in the control group exhibited a significant decrease after ovariectomy, more marked after the administration of exogenous E2. Hence, the latter reduction is in accordance with the restoration of CYP450 3A activity and the induction of CyA metabolism by rat liver microsomes [13] after the administration of E2. The higher amounts of CyA metabolites in control males than females confirm the predominance of native CyA over metabolites in rat blood [26] but the relative proportions in the liver suggest that an accumulation process may exist in this organ. Only the administration of E2 to OVx females significantly decreased the relative abundance of metabolites in the liver (35.18 ± 7.68% of total CyA determined). So then, the levels of CyA metabolites determined by p-FPIA in the liver and blood do not justify the changes in parent drug blood levels from the standpoint of drug metabolism. However, polyclonal FPIA only shows cross-reactivity with parent CyA and some of its metabolites but not with phase two metabolites. Phase II metabolism is another mechanism contributing to CyA clearance in humans [27], although it has not yet been proven in rats. However, the sex-dependency of glucuronidation in rats [19] and the significant amounts of a polar unidentified metabolite found by Wagner et al. [28] after CyA administration to rats make it tempting to speculate that the formation of phase two metabolites in rats could help in the interpretation of the results obtained, particularly with females.

The lower CyA levels found in OVx rats may also be brought about by the slight but significant increase in their body weight upon gonadectomy since the post mortem examination evidenced an increased content in fat thereby suggesting that the distribution volume of CyA might be enlarged. Drug distribution into highly irrigated tissues followed the same relative pattern in control male and female rats as shown by the tissue to blood partition coefficients. In fact, this is consistent with previous results describing that the values of haematological and biochemical parameters that might exert a significant influence showed no gender dependency in normal rats [14]. The highest K_p values always corresponded to the liver pointing towards a major accumulation site for CyA followed by the spleen and the kidneys, independently of animal gender or hormonal status. It is well known that gonadectomy and sex-hormones may induce significant changes in the metabolism of lipoproteins, which are an important binding site for CyA in plasma. Hence, our findings are consistent with previous data indicating that the hepatic extraction of CyA is high and generally non-limited by its binding to blood components [26, 29]. On the contrary, the more extensive distribution of CyA into the spleen and kidneys of ORCHx and ORCHx + TT males as well as the diminished distribution observed into the same organs of OVx and OVx plus E2 treated females may be associated to these lipoprotein changes. Indeed, lipoproteins exert a strong influence on CyA tissue distribution [30]; particularly, HDL and LDL lipoproteins reduce the uptake of CyA by the isolated perfused rat kidney [31]. Finally, the effect of the P-glycoprotein (P-gp) cannot be neglected due to most of the drugs interacting with the P-gp are also substrates for the CYP450 3A subfamily such as CyA [32]. Male rats consistently express several-fold lower levels of P-gp than females [32, 33]. Therefore, the lower CyA blood and tissue levels found in female rats may result from a lower oral bioavailability due to a more pronounced exorption of drug by the enterocytes. The expression of the P-gp in rat canalicular membrane vesicles [34] may also contribute making the biliary excretion of CyA compounds faster in females. Furthermore, the lower tissue concentrations achieved in females are in agreement with the lesser metabolite amounts produced. However, the interaction of several sex-hormones derived compounds (TT and E2 conjugates) with the P-gp in rat canalicular membrane vesicles [34], the induction of the multidrug resistance gene by E2 in rats [35] and the lack of available data about the effect of gonadectomy on the P-gp expression in rats makes difficult to infer reliable conclusions from our results. Furthermore, CyA itself exerts a significant effect on sex-hormones secretion [36], lipoprotein profiles and even on CYP450 3A expression in rats [37, 38] that may contribute to explain our results.

Nonetheless, it becomes evident that gonadectomy exerts an important effect on CyA pharmacokinetics, particularly in females. Although the results obtained in rats cannot be directly extrapolated to humans, this effect should be carefully evaluated in gonadectomized transplant patients since it may affect the therapeutic efficacy of the drug and its toxicity. Furthermore, since CyA is mainly cleared through metabolism by the predominant CYP450 3A, it should be noted that the pharmacokinetics of many other drugs, undergoing the same metabolic pathway, may be affected by gonadectomy as well. Additional *in vitro* and *in vivo* studies are needed to adequately explain these findings.

4. Experimental

4.1. Animals and surgical procedures

Experiments were conducted on adult male (10 weeks-old) and female (40 weeks-old) Wistar rats initially weighing 240 ± 50 g (mean $\pm$ SD) obtained from the central stabulary of the University (Homologation number E.C. 28005-22A). Due to different growth curves for males and females, the age of rats resulted involved as well. Nonetheless, it had been previously determined that age did not influence the values of important pharmacokinetic parameters (AUC, MDRT or V_{ss}) in female rats [14]. The animals were maintained on a 12 h dark-light cycle, with free access to food and tap water. Five rats were randomly assigned to each of ten groups as follows:

- Control male (MV) and female (FV) rats receiving the vehicle of CyA.
- Control gonadectomized males (CORCHx + TT) and females (COVx + E2) receiving hormone supplementation but no CyA.
- Normal (sham operated) male (M) and female (F) rats receiving CyA.
- Orchiectomized male rats (ORCHx) and ovariectomized female rats (OVx) treated with CyA,
- and finally, orchiectomized male rats (ORCHx + TT) and ovariectomized female rats (OVx + E2) that received CyA and hormone supplementation, simultaneously.

A mixture of diazepam (Valium®, 2 mg/kg b.W.) and ketamine (Ketolar®, 10 mg/kg b.W.) was intramuscularly administered as anesthetic agent during orchiectomy, ovariectomy and sham operation.

Orchiectomy was performed via a small median skin incision of about 1 cm at the tip of the scrotum. Then, the subcutaneous connective tissue was cleared, and a small 5 mm cut was made into each sac in order to make a ligature of the vas deferens and the spermatic blood vessels prior to the removal of the testis and the epididymis. Ovariectomy was performed by making a midline dorsal skin incision half way between the middle of the back (the hump) and the base of the tail. After, muscle incisions were made and the ovary was pulled out through. Then, the periovarian fat was removed and the function between the Fallopian tube and the uterine horn, together with all accompanying blood vessels was severed with a single cut and the horn returned into the abdominal cavity.

4.2. Drug treatment and extraction of biological samples

Animals were housed in metabolical cages for 24 h after operation to allow for recovery with free access to food and tap water. The effect of sex-hormone replacement was studied in gonadectomized male rats (groups CORCHx + TT and ORCHx + TT) given testosterone propionate (TT, 1 mg/kg per day, im. injection) (LEO Laboratories, Spain). M and ORCHx rats also were i.m. injected for the same period of time with the vehicle of TT (ethanol 100 µl/day). Gonadectomized female rats of the groups (COVx + E2) and (OVx + E2) were processed in the same way but they received estradiol (E2, Sigma Chemical, Spain) by im. injection at a dose of 10 µg/kg daily as hormonal replacement therapy. F and OVx rats also received the vehicle im. (ethanol 100 µl/day). Both hormonal treatments were given for the whole study period, starting one day after surgery. The restoration of the estrous cycle in gonadectomized females was complete after five days of E2 treatment, as shown by the proportion between nucleated and cornified cells in vaginal smears. Therefore, after this period the animals were given an emulsion of the oily CyA solution in whole milk (10 mg/kg per day) or the vehicle. In all cases, volumes under 500 µl were administered by gastric gavage at 10 a.m. for 3 days. The results by Pell et al. [26] suggest this treatment period is long enough to reach steady-state conditions by the oral route. In the morning of the fourth day ($\sim$10 a.m.), animals were weighed and, under isoflurane anesthesia, blood was collected from the lower aorta into glass tubes containing 7.5% EDTA (sodium salt) for whole blood CyA quantitation. After animal exsanguination, tissues were perfused with 60 ml isotonic NaCl at 37 °C via the catheter inserted in the abdominal aorta for blood collection. Once residual blood was eliminated, the organs (liver, kidney and spleen) were removed and weighed. Then, a 300 mg portion (wet weight) from each one was homogenated in 2 ml acetonitrile (MeCN) and 120 µl trichloroacetic acid (10% in methanol) were added to complete protein precipitation. After vortexing, the samples were centrifuged at 4,500 rpm during 20 min. A 2 ml aliquot of the supernatants was evaporated under a nitrogen stream and mild heating (maximum 40 °C) and stored at -20 °C until analyzed. Finally, drug content in each tissue was determined after resuspension of the dried extracts in variable volumes of MeCN (500 µl for the liver and 300 µl for the rest of tissues) and centrifugation at 4,500 rpm during 10 min.

4.3. Analytical procedures

Blood and tissue levels of CyA were determined by a specific monoclonal antibody fluorescence polarization immunoassay (m-FPIA, TDx Abbot Laboratories). Blood samples were analyzed by adding 50 µl MeCN to 100 µl of the sample specimen. MeCN was included in order to increase drug extraction in tissue samples. Afterwards, the solubilization (50 µl) and precipitation reagents (300 µl) were added and the samples centrifuged at 11,000 rpm during 4 min. CyA concentrations in the different tissues were analyzed following the same protocol, but 50 µl of the resuspended residue in MeCN were mixed with 100 µl of 0.9% NaCl. Cyclosporine concentrations were converted to ng/g wet weight for comparisons to blood concentrations. Tissue to blood partition coefficients (K_p) were calculated according to the following equation:

$$K_p = \frac{C_t}{C_b \cdot (1 - E)}$$

where C_t (ng/g) and C_b (ng/ml) are the tissue and blood concentrations, respectively, and E stands for the tissue extraction ratio. The E value was calculated from Cl/Q_H where Q_H represents the hepatic blood flow (11.80 ml/min). As the liver is the major determinant for drug elimination E was set equal to zero in the rest of tissues.

Total CyA derivatives present in blood or accumulated in the liver were determined using a fluorescence polarization immunoassay with polyclonal antibodies (p-FPIA, TDx Abbot Laboratories). Drug assays were carried out on the supernatants by means of a TDx analyzer (Abbot Laboratories). Then, the concentrations of CyA metabolites were approximately estimated by a simplistic but useful method, as the difference between the drug levels measured with the nonspecific p-FPIA and the more specific m-FPIA methods. Calibration curves with the monoclonal and polyclonal antibodies were performed in blood and tissues showing that the percent recovery of CyA in blood samples ranged from 95 to 102%, while it approached 83.62 ± 12.15% in tissue samples. The within-day and between-day coefficients of variation for the same batch of reagents did not exceed 5% and the limit of quantification was 25 ng/ml and 65 ng/ml for m-FPIA and p-FPIA, respectively.

4.4. Statistical methods

Comparisons were performed by the Kruskal-Wallis test (comparisons between more than two groups) or the Mann-Whitney test (comparisons between two groups). A level of statistically significance $p < 0.05$ was considered. Results are expressed as mean $\pm$ standard deviation unless otherwise stated.

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