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Induction of Cell Cycle Arrest in Tumor Tissue And Bone Marrow Of Grafted Mice Treated with Androgen and Antiandrogen.
Running title: Effect of androgen and antiandrogen on leukemia cell line

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ABSTRACT: *Background:* These works were designed to investigate the influence of androgens (by castration and testosterone treatment) on growth and development of murine leukaemia cell line P388 *in vitro* and *in vivo*. To approach the mechanism of this hormone, the cell cycle was analysed in bone marrow cells and tumour tissue in vitro and on murine leukaemia cell line (P388) in culture. The anti-androgen CA was used to provide additional information concerning the androgen receptor in leukaemia cells.
Materials and methods: The effect of Depo-Testosterone (DT) and Cyproterone-Acetate (CA) were studied on growth tumour and cell cycle progression, in bone marrow and tumour tissue of intact or castrated P388 tumour-bearing BDF1 mice. In parallel the effect of various concentrations of DT (10^{-8}, 10^{-7}, 10^{-6}, 10^{-4} M) was investigated on the proliferation and cell cycle of P388 leukaemia cell line *in vitro.*
Results: In P388 tumour-bearing mice, DT (0.5mg/100g body weight) treatment reduced strongly the weight and the appearance rate of tumour in non-castrated (NC) and castrated animals. In NC animals, the cell cycle analysis showed a significant decrease in the number of cells in S phase in tumour tissue under DT or CA treatment. Same results were obtained in bone marrow with DT only. The cells accumulate into G2/M and G0/G1 phases respectively. In vitro testosterone can inhibit the proliferation of leukaemic cells with a pharmacological dose of 10^{-7}M. This growth inhibition was associated with cell cycle arrest in G0/G1 phase. This effect was dose and time dependent.
Conclusion: The data demonstrated that both *in vivo* and *in vitro*, testosterone prevent the growth of P388 leukaemia cells and induce changes in their cell cycle. The similar effect of CA and DT on tumour growth inhibition, in cell cycle of bone marrow and tumour tissue may suggest differences between androgen receptors of sexual organs and leukemic cells.

KEYWORDS: Testosterone, Cyproterone-Acetate, P388 leukaemia cell line, Cell cycle, Tumour tissue, Bone marrow cells.

1 INTRODUCTION

A characteristic of acute leukaemia cell is to loose the ability to differentiate into mature, functional cells and remain in a high proliferative status over their normal counterpart. One possible approach to treat patients with acute leukaemia is to use agents that can induce differentiation of leukaemic cells and inhibit their proliferation [1], or by cell cycle alteration that leeds to apoptosis [2]. Recent studies reported that E2 inhibits apoptosis in different cell types, whereas androgens have been found to induce apoptosis [3].

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The works of H. Everaus [4] proved that abnormalities in the immune system observed in CLL (Chronic Lymphocytic Leukaemia) may be connected with an imbalance of endocrine regulation. The underlying mechanism for disorder in T cell function in CLL is still unknown.

In acute non-lymphocytic leukaemia (ANLL), marrow transplantation offers some survival advantage. Among therapeutic agents, the sex steroids have been reported to have some effect on ANLL [5]. A study [6] about two homo-azo steroids (Androgens and Oestrogens derivatives class) indicated that these two agents exhibited stimulatory effects on the proliferation of ANLL cells, especially in blast cells with low degrees of differentiation. However, the mechanisms implicated in this regulation were not clear.

The 2beta-(4-methylpiperazinyl)-5alpha-androstane-3alpha, 17beta-diol was recently reported for its ability to inhibit the proliferation of human leukemia HL-60 cells [7].

Cell cycle control in G1 phase has attracted considerable attention in recent cancer research, because many of important proteins involved in G1 progression, or G1/S transition have been found to play a crucial role in proliferation, differentiation, transformation and programmed cell death (apoptosis) [8].

Cyproterone Acetate (CA) is a potent, synthetic steroid that possesses anti-androgenic, anti-oestrogenic, anti-gonadotrophic and anti-progestational activity. Its anti-androgenic activity is due to competitive inhibition of androgen binding receptor [9].

Based on these findings, we investigated the effect of testosterone (DT) on growth tumour and proliferation of lymphoid leukaemia cell line (P388) *in vivo* and *in vitro*. We have followed the cell cycle progression in bone marrow, tumour tissue and in leukaemia cell line in culture, with or without DT. Furthermore the effect of CA was studied to provide additional indirect information on androgen receptors.

2 MATERIALS AND METHODS

2.1 ANIMALS

BDF1 male mice (C57BL/6 x DBA/2) F1, 3 weeks old, (IFFA-CREDO, Les Oncins, France), were housed in air-conditioned room (temperature 21±2°C) in standard cages each housing 5 animals. Food and water were available *ad libitum*. The mice were kept on a 12h light-dark cycle (L/D, 12:12 hours).

2.2 CASTRATION

Male mice were castrated at four weeks old. They were anaesthetised with ether and castrated by making an abdominal incision, removing the testes and closing the incision with 6-0 silk sutures. As controls there were two groups: sham-operated and non-operated (intact) animals. It was found that the sham-operated group was indistinguishable from non-operated controls; hence the data from these two groups were pooled.

2.3 STEROIDS

Depo-testosterone (DT, Testosterone17 beta-cypionate, Sigma-Aldrich, L'IsleD'AbeauChesnes, France) or Cyproterone Acetate (CA, generously provided by Schering, West Berlin, Germany) in sterile peanut oil (PNO) were administered subcutaneously in mice at a dose of 0.5mg/100g body weight, in a final volume of 0.2 ml. The same volume of PNO was injected in castrated and in intact control group. DT was administered to animals on alternate days for a period of two weeks. CA was given to mice on five consecutive days of each week over a period of two weeks. Steroids were injected in treated animals one week after castration. Dose, route and frequency of DT and CA administration were chosen according to the works of Ahmed *et al.* [10]. The experimental groups are summarised in Table I. 7 to 12 animals per batch were used.

2.4 CELL LINE

P388 leukaemia cell line, from a DBA/2 mouse, was obtained from Flow, France. Cells were maintained in culture in Fisher medium supplemented with 5% foetal bovine serum, 1% glutamine (2mM) and 1% penicillin-streptomycin, at 37°C in a humidified atmosphere of 5% CO_2. The cells were counted with an automate (Coulter Argency, France). Trypan blue exclusion test indicated that the viability always exceeded 90%.

2.5 TRANSPLANTED MICE

The P388 cells were injected subcutaneously at 10^5 cells per mouse and the mice were examined daily to monitor survival and tumour growth. The P388 tumours could only be followed for approximately two weeks after which the animals developed leukaemia and died. For that reason, the mice were distributed in two batches and killed after 15 days or 30 days after grafting. Control mice were injected with Fisher medium, or with peanut oil, no difference was observed between the two batches. In grafted mice, the injections of drug (DT and CA) start in the same day of inoculating mice with leukaemia cells.

2.6 IN VIVO PROTOCOLS

There are 2 batches of mice castrated and none castrated (NC or intact). Each batches containing 4 groups: untreated; grafted with leukaemia cell line; transplanted treated with DT; transplanted and treated with CA.Seven to twelve animals were used per experimental condition.

2.7 DT *IN VITRO* EFFECT

P388 leukaemia cells were seeded at 2-5 x 10^5 cells/ml in culture flask, in Fisher medium supplmented with DT diluted in ethanol; vehicle was less than 0.05%. The DT (10^{-8}, 10^{-7}, 10^{-6}, 10^{-4} M) was added to the cell culture on day 0 and the cultures were monitored for 3 days. Numeration of cells was performed by the Coulter counter automate. The viability was determined by trypan blue exclusion test and by flow cytometry.

2.8 PLASMA TESTOSTERONE

Mice were killed by cardiac exsanguination. The blood was collected in heparinised syringes and the plasma was separated by centrifugation. The plasma testosterone concentration was measured by radioimmunoassay (Dr.Baudin, Department of Nuclear Medicine, CHU-Nimes).

2.9 CELL PREPARATION

Bone marrow cell suspensions were prepared by flushing phosphate buffered saline (PBS) through the femur. The thymus, spleen, seminal vesicle and tumour tissue were removed from each mouse and weighed. The weight of the organs was expressed in mg/100g body weight. The tumour tissue was placed in Petri dishes containing PBS; cells were then suspended by fine needle aspiration. The cells were stained with propidium iodide (IP) by the 3 steps method of Vindelov*et al.* [11].

2.10 FLOW CYTOMETRIC ANALYSIS

Samples were run in a flow cytometer (cytofluograph IIS, OrthoDiagnostic Instruments, Westwood, Massachusetts, USA) equipped with an innova 90-5 argon ion laser (Coherent PaloAlto, California, USA) operating at 488nm and 500mW in light-regulated mode. The cytometer was calibrated with fluorescent microbeads before each run. Data from the forward angle scatter and fluorescence parameters (peak and area signals of fluorescence emission) were collected by user-defined protocol and stored in an Ortho 2151 computer system (Ortho Diagnostic Instruments) in list mode.

The DNA content histograms (red fluorescence emission area versus number of cells per channel) were established from 20,000 events gated on 'red fluorescence area peak' cytograms in order to discriminate doublets. The proportion of cells in the G0/G1, S and G2/M phases of the cell cycle was calculated from the histograms by the parametric method.

2.11 STATISTICAL ANALYSIS

The data were analysed with student's t-test. A p value of less than 0.05 was considered significant.

3 RESULTS

3.1 IN VIVO STUDY

3.1.1 TUMOUR GROWTH *(TABLE 1)*

The percentage of mice that developed tumours was higher in castrated mice (70%), than in non-castrated (NC) mice (62.5%). The NC mice survived longer (87.5%) than castrated mice (50%). Compared to non treated NC mice, the DT and CA injection reduced strongly the tumour growth; less than 10% showed a tumour. A lower tumour decrease was observed in castrated mice treated by steroid hormones (40% for DT; 50% for CA).

3.1.2 TUMOUR WEIGHT *(TABLE 1)*

In castrated mice the tumour weight was more important than in NC grafted mice (12.5±4.5 against 9.5±2.3, $p<0.05$). The injection of DT and CA reduced significantly ($p<0.05$) the tumour weight in NC mice. In castrated mice, though there was a decrease in tumour weight under steroid treatment, the difference was not significant compared to non treated animals.

3.2 ORGANS WEIGHT *(TABLE 1)*

3.2.1 SEMINAL VESICLE

The weight of the seminal vesicles was initially determined in NC mice and in castrated mice. The castration and CA administration reduced significantly ($p<0.01$) the weight of the seminal vesicles compared to control. DT treatment induced a significant ($p<0.01$) increase in the weight of seminal vesicle in intact and in castrated mice.

3.2.2 THYMUS

As expected, the weight of the thymus in castrated mice was greater than in intact mice ($p<0.001$). No significantly difference was observed between transplanted mice and control mice. Compared to control, the weight of thymus decreased significantly both in intact and in castrated mice when either DT ($p<0.001$) or CA ($p<0.01$) were injected.

3.2.3 SPLEEN

The weight of the spleen was significantly higher in P388 tumour-bearing NC mice than in controls ($p<0.01$). There was no significant change in the weight of the spleen after DT injection.

3.3 PLASMA TESTOSTERONE LEVEL

The plasma testosterone concentration was significantly decreased ($p<0.001$) in castrated mice. Compared to control, the plasma testosterone level was increased after DT injection in NC ($p<0.01$) and castrated mice ($p<0.001$). Whereas, the CA treatment decreased significantly ($p<0.001$) the testosterone level in NC transplanted mice, no change in testosterone level was observed in castrated ones, since it was already low (Table 1). There was a significant regressive curve between tumour weight and testosterone level ($r=0.9$, $p<0.01$, Figure 1).

3.4 CELL CYCLE ANALYSIS

3.4.1 BONE MARROW

As bone marrow cells have been collected under the same conditions, the number of cells/ml (Table 1), was almost the same between NC and castrated grafted mice. A decrease was observed after DT and CA treatment with a higher effect with DT on NC animals and with CA on castrated mice.

Compared to non grafted animals, in leukaemic NC and castrated mice, the cell cycle analysis showed a significant ($p<0.05$) increase in the number of cells in S phase and a significant ($p<0.05$) decrease in G2/M phase in bone marrow (Figure 2). In NC leukaemic mice, DT ($p<0.01$) and CA ($p<0.05$) treatment reduced the percentage of cells in S phase and increased

the relative number of G2/M cells. DT and CA injection have no effect on cell cycle of leukaemic castrated mice compared to non-treated mice.

3.4.2 TUMOUR TISSUE

Compared to untreated animals, the DT and CA administration increased significantly the percentage of G0/G1 cells (Figure 3) which was balanced by a diminution in S phase. The percentage of proliferating cells showed a significant reduction in both NC ($p<0.01$) and castrated ($p<0.05$) mice. In DT treated castrated mice, a correlation was observed between the percentage of proliferating cells ($r=0.86$; $p<0.01$), the relative number of cells in G0/G1 phase ($r=0.85$, $p<0.01$) and tumour weight (Figure 4).

3.5 IN VITRO STUDY (P388 CELL CULTURE)

3.5.1 CELL GROWTH

Figure 5 shows the growth curve of P388 cells, with a doubling time of approximately 24 hours. DT treatment greatly reduced cell growth; its effect was time and dose dependent. Growth inhibition started after 24 hours of culture with the two highest DT concentrations (10^{-6} and 10^{-4} M).

3.5.2 CELL CYCLE ANALYSIS

In exponentially growth culture, P388 cells were mainly in G0/G1 and S phase (57.9±3.1 and 35.9±3 % respectively), the proportion of cells in G2/M phase was low (7.6±2 %) (Figure 6). According to growth inhibition, DT treatment induced a significant decrease in the number of cells in S and G2/M phases and an increase of cells in G0/G1 phase. DT treatment efficacy was observed up to 10^{-7} M concentration.

4 DISCUSSION

These works were designed to investigate the influence of androgens (by castration and testosterone treatment) on growth and development of murine leukaemia cell line P388 *in vitro* and *in vivo*. To approach the mechanism of this hormone, the cell cycle was analysed in bone marrow cells and tumour tissue in vitro and on murine leukaemia cell line (P388) in culture. The anti-androgen CA was used to provide additional information concerning the androgen receptor in leukaemia cells.

The DT and CA treatment reduced strongly the weight and the rate of tumour in BDF1 NC or castrated mice grafted with murine leukaemia cell line (P388). Cell cycle analysis showed that DT or CA induces a decrease in the number of S phase cells in bone marrow and tumour tissue; the cells accumulate in G2/M phase in bone marrow and G0/G1 phase in tumour tissue in NC mice.

A recent report demonstrated that testosterone inhibits the *in vivo* tumourigenic properties of the 1246-3A cells. Castrated male mice receiving injections of 1246-3A cells developed larger tumour at a higher frequency than sham-operated animals. Administration of testosterone to castrated male mice resulted in a dramatic decrease in tumour development [12]. In human, a decrease in serum level of DHEA may be associated with patients who have some clinical subtypes of ATL [13]. The 17β-estradiol/ Testosterone ratio was considerably higher in men with CLL [14]. Chronic lymphocyte leukaemia (CLL) is the most common lymphoproliferative disease. Normal hematopoiesis is a tightly regulated process involving a balance between signals that stimulate and those that inhibit the proliferation and differentiation of hematopoietic progenitors. In leukaemia patients there is a perturbation of these controlling elements, resulting in overgrowth of leukaemic cells in bone marrow and spleen cells [15].

In our results, the cell cycle analysis showed that testosterone or CA treatment decreased the proliferation observed in bone marrow cells of transplanted mice with cell cycle arrest at the G2/M phase. Previous studies showed that androgens can directly stimulate healthy bone marrow cells to proliferate and differentiate [16]. In healthy mice, DT decreased strongly the percentage of bone marrow cells in G2M phase [17] whereas in our experiment with P388 tumour-bearing mice the sex hormone increased the relative number of bone marrow cells in G2/M phase. The bone marrow was probably overrun by leukaemia cells. In fact, in rats inoculated with leukaemic cells, the fraction of RRM 124+ cells (the RRM 124 antibody

identifying the leukaemic cells) was significantly increased in bone marrow and spleen [18]. However, DT treatment acted differently in the proliferation of healthy bone marrow or overrun with leukaemia cells.

In the present findings, the cell cycle analysis in tumour tissue showed that DT decreased both the S and the G2/M phases, with accumulation of cells into G0/G1phase. CA treatment gave same results as DT treatment. There is a correlation between tumour weight and the percentage of cells in G0/G1 (Figure 4). These data showed that testosterone prevent the proliferation of leukaemic cells both in bone marrow and tumour tissue.

CA decreases seminal vesicle weight in the intact and castrated mice. It is one of the most active antiandrogenic steroids blocking the peripheral action on sexual organs of both endogenous and exogenous testosterone [19]. The similar effect of CA and testosterone in the reduction of the weight and rate of tumour, and a decrease in the proliferation of cells in bone marrow and tumour tissue suggests a similar specificity in the biological activity of these two steroids.

Three explanations might be put forward. Firstly, that CA and DT may act through the immune system on leukaemia cells. In fact, testosterone and CA increases the % of CD4+ thymocytes and CD8+ splenocytes [19]. The inhibition of tumour growth is strongly associated with the level of CTL (Cytotoxic T lymphocyte) activity present in CD8+ cells derived from the spleen. The level of CD8+ CTL activity is directly correlated with the degree of inhibition of tumour growth [12, 20]. Second, testosterone could inhibit cell proliferation by blocking vessel development, by enhancing apoptosis in vascular endothelial cells [3]. Third, these two steroids act directly on leukaemia cells through a common receptor. To check the direct action of testosterone on leukaemia cells, we investigate the growth of murine leukaemia cell line (P388) and studied the modulation of cell cycle changes with this hormone.

Our experiments showed that testosterone can inhibit the proliferation of leukaemic cells with a pharmacological dose (10^{-7}M). Lower dose was ineffective. This growth inhibition, dose and time dependent, was associated with cell cycle arrest; P388 cells accumulates in G0/G1 phase. Our finding are in agreement with Mossuz*et al.* (4) who reported that the testosterone can inhibit the proliferation and the clonogenic potential of the human monoblasticleukaemic cell line U937. The growth inhibition was associated with cell cycle arrest, U937 cells accumulating in G2/M phase.

Many anti-tumoural compound act by the way of cell cycle arrest, leading to accumulation of cells in G2/M or G0/G1 phases and cell growth inhibition [21]. In P388 murine leukaemia cells, a potent anti-tumour marine steroid (YTA0040) suppresses nucleic acid and protein synthesis. Cell cycle analysis revealed that this steroid blocked the cells into S phase leading to arrest in the late G1 phase [22].

The novel aminosteroid 5alpha-androstane (HY) inhibit the proliferation of the human promyelocytic leukaemia cell line HL-60. This aminosteroid HY was a derivative of 5 alpha-androstane and posses some structural similarities with an androgen steroid [23]. Methoxyestradiol (2-ME2) markedly suppressed proliferation of human acute T lymphoblastic leukemia CEM cells in a time- and dose-dependent manner. Exposure to 2-ME2 led to G2/M phase cell-cycle arrest, which preceded apoptosis characterized by the appearance of a sub-G1 cell population [24].

Cyproterone Acetate (CA) is a potent synthetic steroid exhibiting anti-androgenic effect, blocking the peripheral action on sexual organs of both endogenous and exogenous testosterone [19] and also progestational activity [9]. The progesterone is a potent cytostatic agent [25].

DT and CA could act through a receptor common to steroid compounds, but different from the androgenic receptor found in sexual organs. Specific androgen receptors were detected in non-Hodgkin's lymphoma cells [26].

Data demonstrated the presence of binding sites in acute lymphoid and myeloid leukaemia, named Type II ER binding sites (type II EBS) [27].

The works of DY Jun and al (2008) [28] reported that the cytotoxicity of 17α-E2 toward Jurkat T cells is attributable to apoptosis mainly induced in G2/M-arrested cells, in an ER-independent manner, via a mitochondria dependent caspase pathway regulated by Bcl-2.

The present *in vivo* experiment showed that testosterone inhibits tumour growth and cell proliferation both in bone marrow and tumour tissue. *In vitro* study demonstrated that testosterone can also inhibit the proliferation of the murine leukaemic cell line P388 in a dose and time dependant manner.

The similar effects of DT and CA give two informations : the androgenic receptor that mediates these steroid effects is different from the receptor of sexual organs and this receptor is probably specific to steroid compounds.In fact, the cytotoxic effect of 17α-E2 against human leukemias Jurkat T and U937 cells was not suppressed by the ER antagonist ICI 182,780 [29].

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ANNEX

Table I: Effect of DT or CA on the plasma testosterone concentration (nmol/l), thymus, spleen, seminal vesicle (mg/100g) and tumour (mg) weights of non-castrated (NC) and castrated mice.

		Thymus mg/100g ± sem	Spleen mg/100g ± sem	Seminal vesicle mg/100g± sem	Testosterone nmol/l ± sem	Tumour weight mg ± sem	Tumour appearence %	Survival %	Bone cells/ ml x10^6
NON-CASTRATED	Untreated	246 ± 68.1	336.5 ± 24.3	372 ± 63.2	22.4 ± 1				2.5±0.3
	TM	200.1 ± 46.1	437.1 ± 68.9 **	374.6 ± 68.9	20.5 ± 2	9.5 ± 2.8	62.5	87.5	3.3±1.1
	DT	86.9± 36.8 **++	508.9 ± 17.6**	514.8 ± 72.0 **++	50.8 ± 11.1 **++	6.5 ± 2.3+	8.3	83.3	0.95±0.5 p<0.005
	CA	153.9 ± 35.9 **+	290.2 ± 39.1 *++	240.5 ± 70.6 **++	5.8 ± 1.2 **++	6.8 ± 2.8+	9	80	1.6±0.8 p<0.05
CASTRATED	Untreated	330.1 ± 36.8°°	384.2 ± 47.9	228.1 ±67.9°°	0.03 ± 0.01°°				3.2±0.5
	TM	300.0 ± 25.5°°	400.5 ± 30.8	230.0 ± 65.2	0.02 ± 0.008	12.5 ± 4.5	70	50	3.4±1.6
	DT	170.0 ± 50.5 p < 0.001	470.9 ± 60.0	480.3 ± 76.3 p <0.01	20.5 ± 7.5 p< 0.001	8.5 ± 3.5	40	40	1.15±0.7 p<0.05
	CA	200.5 ± 6 0.8 p<0.01	350.0 ± 58.9	220.9 ± 75.9	0.03 ± 0.005	9.0 ± 2.4	50	40	0.53±0.3 p<0.05

Values are the means ± SEM. The transplanted mice were compared to animals without tumour. The transplanted DT or CA treated-mice were compared to grafted untreated mice.

TM: NC or castrated transplanted mice
DT: NC or castrated transplanted mice treated with DT.
CA: NC or castrated transplanted mice treated with CA.

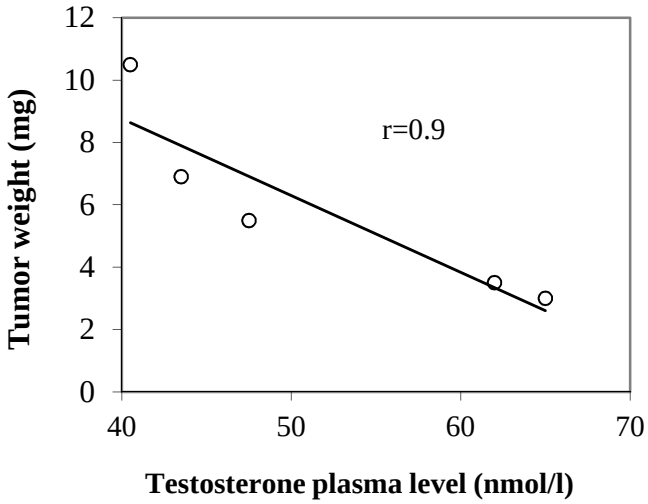

Fig.1. Correlation between the plasma testosterone level and tumour weight (r= 0.9) in NC DT treated mice.

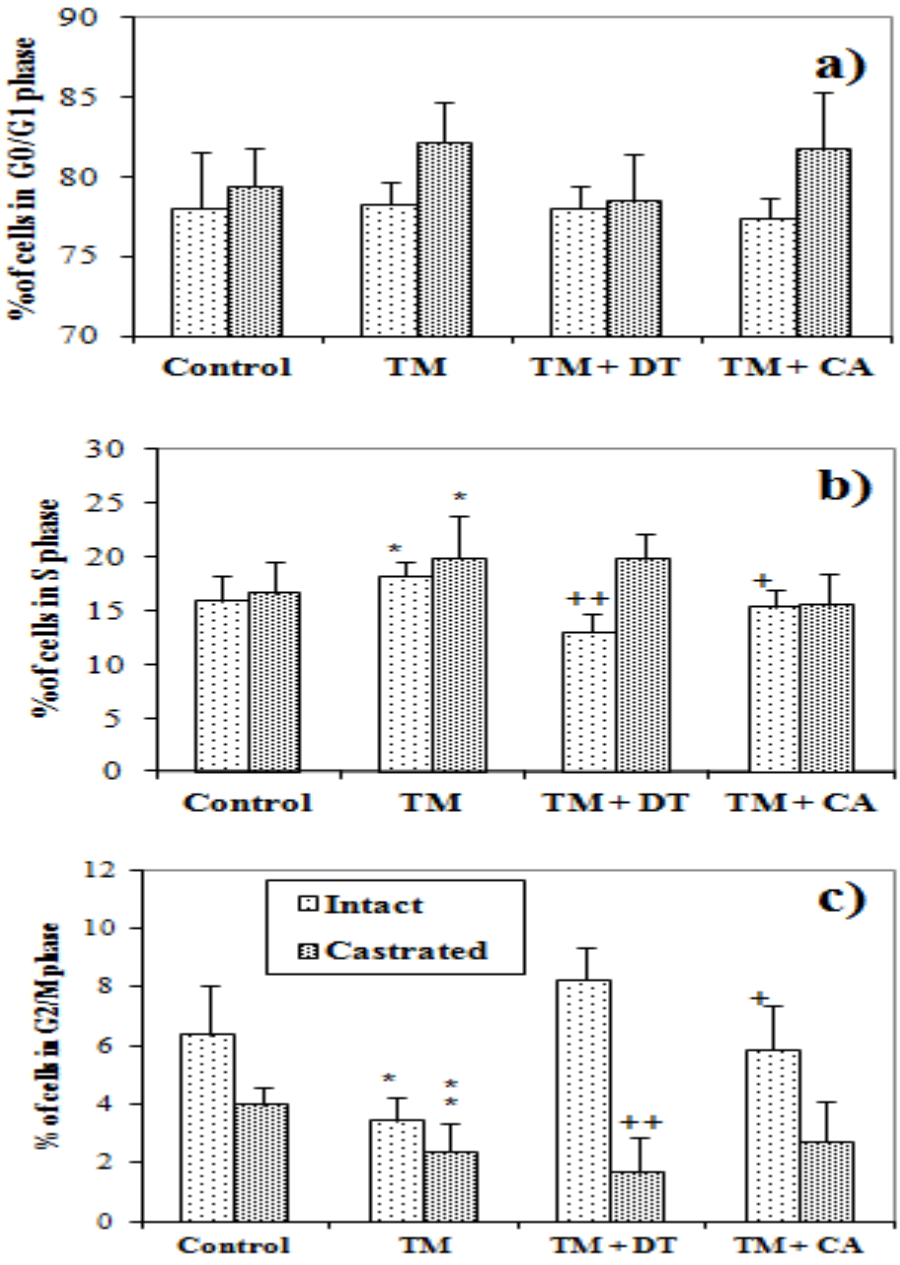

Fig. 2. ***Effect of DT or CA (0.5mg/100g body weight) on the cell distribution among the phases of the cell cycle: G0/G1 (a), S (b), G2/M (c) in bone marrow cells of NC and castrated control mice or with tumour. Data are means ± SEM.***

*p<0.05 compared to NC or castrated mice without tumour.
+ p<0.05, ++ p<0.01, compared to tumour-bearing mice without treatment.

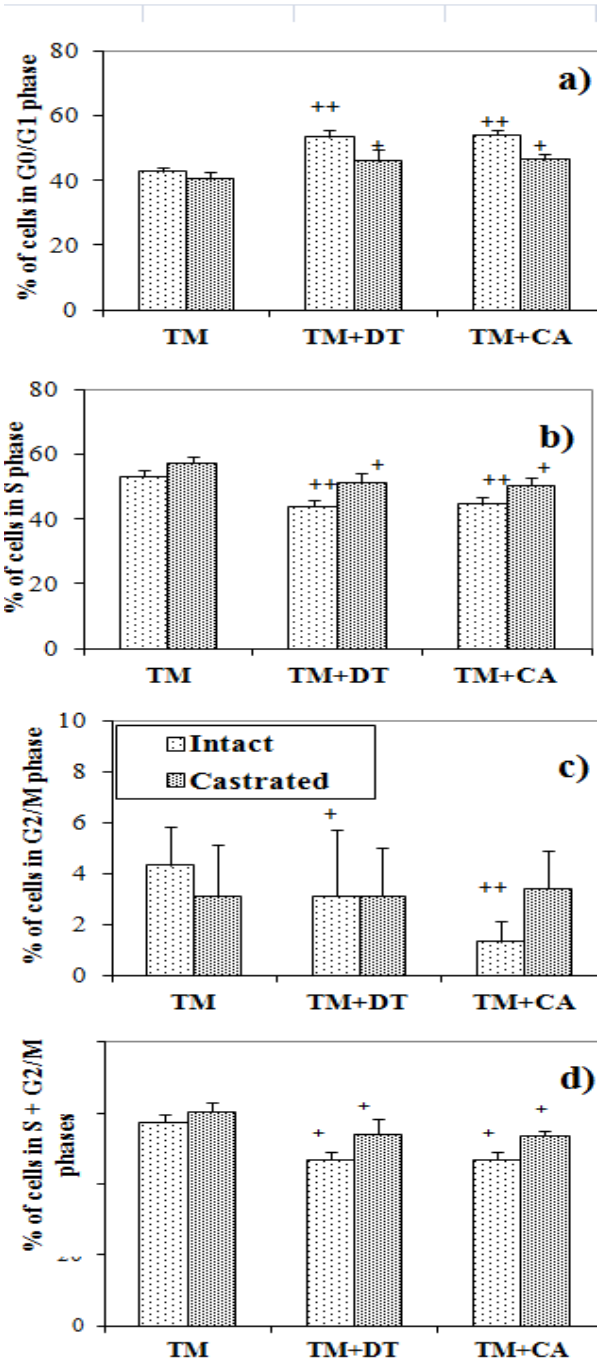

Fig. 3. Effect of DT or CA on the repartition of cells among the phases of the cell cycle (G0/G1 (a), S (b), G2/M (c) and proliferative phase (d) in tumour tissue of NC and castrated transplanted mice.

Data are means ± SEM. + $p<0.05$, ++ $p<0.01$, compared to tumour-bearing mice without treatment.

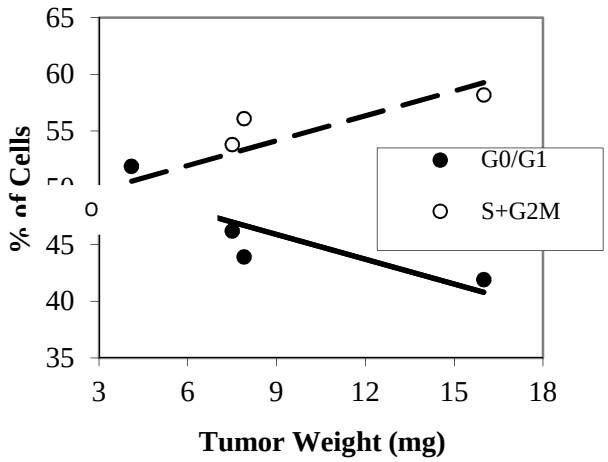

Fig. 4. Regression Curve between the tumour weight and the percentage of cells in G0/G1 phase (r=0.86, p<0.01) and in proliferative phase (r=0.85, p<0.01), in castrated P388 tumour-bearing mice treated with DT.

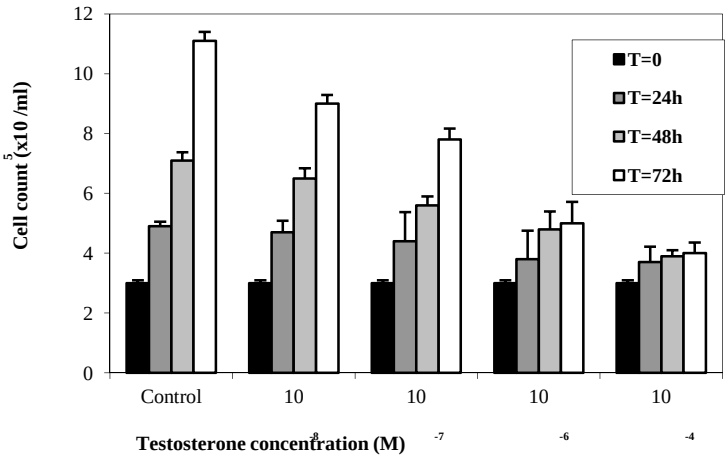

Fig. 5. Effect of DT concentration on P388 cell growth in vitro.

DT was added in the medium at the beginning of the culture. Culture was followed during 3 days. Data are mean cell count ± SEM.

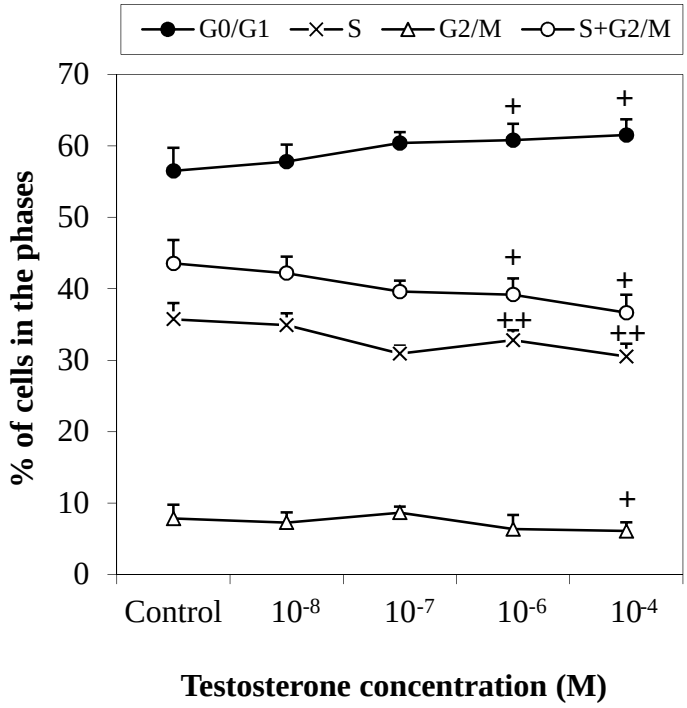

Fig. 6. In vitro effect of DT concentration on the proportion of P388 cells in the different phases of the cell cycle.

Data are mean ± SEM.
+ p<0.05, ++ p<0.01 compared to untreated cells.