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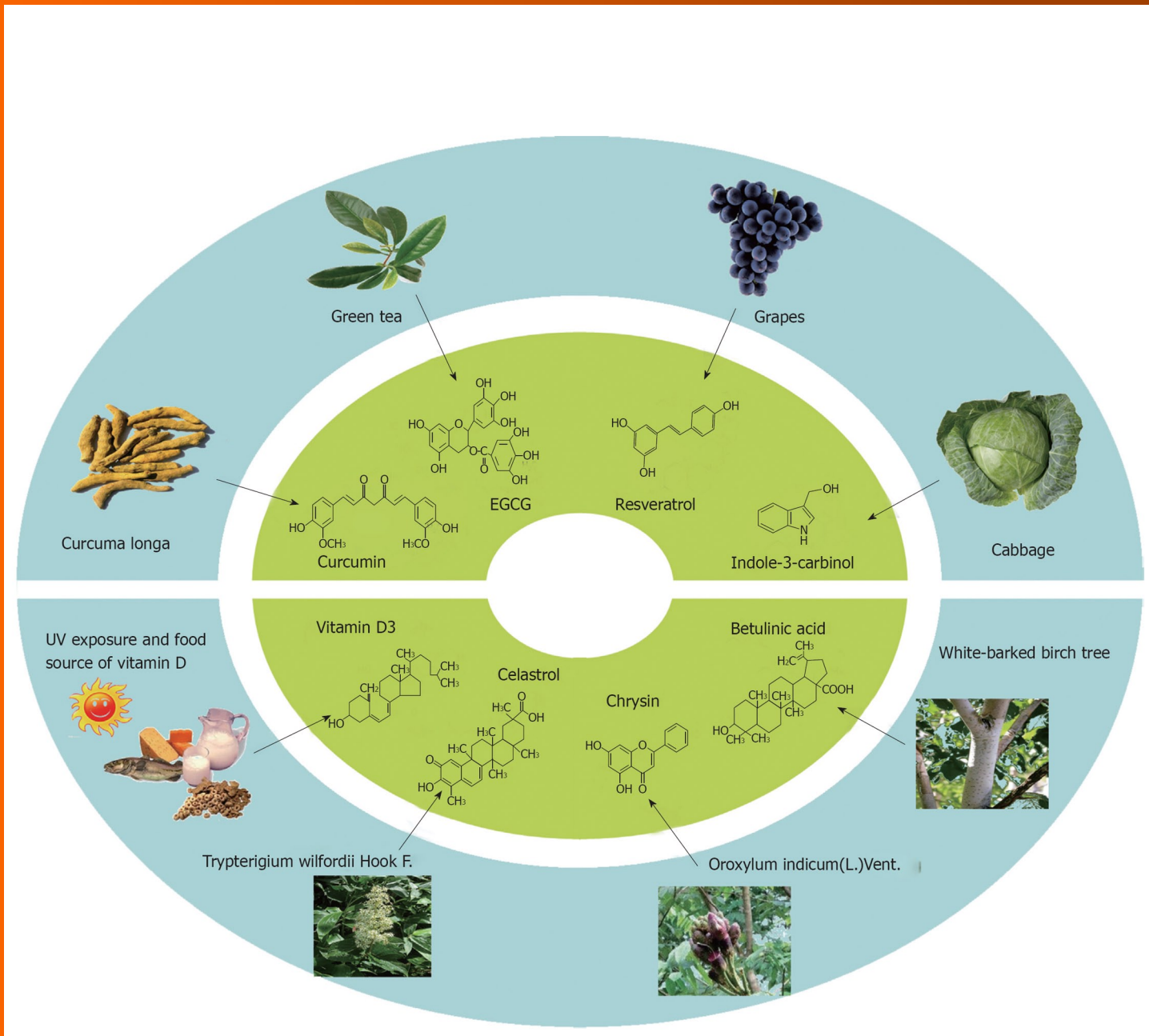

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EDITORIAL

Potential for a pluripotent adult stem cell treatment for acute radiation sickness

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Abstract

Accidental radiation exposure and the threat of deliberate radiation exposure have been in the news and are a public health concern. Experience with acute radiation sickness has been gathered from atomic blast survivors of Hiroshima and Nagasaki and from civilian nuclear accidents as well as experience gained during the development of radiation therapy for cancer. This paper reviews the medical treatment reports relevant to acute radiation sickness among the survivors of atomic weapons at Hiroshima and Nagasaki, among the victims of Chernobyl, and the two cases described so far from the Fukushima Dai-Ichi disaster. The data supporting the use of hematopoietic stem cell transplantation and the new efforts to expand stem cell populations *ex vivo* for infusion to treat bone marrow failure are reviewed. Hematopoietic stem cells derived from bone marrow or blood have a broad ability to repair and replace radiation induced damaged blood and immune cell production and may promote blood vessel formation and tissue repair. Additionally, a constituent of bone marrow-derived, adult pluripotent stem cells, very small embryonic like stem cells, are highly resistant to ionizing radiation and appear capable of regenerating radiation damaged tissue including skin, gut and lung.

Key words: Nuclear accident; Acute radiation syndrome; Radiological casualties; Stem cell transplantation; Cellular therapy; Emergency response; Ionizing radiation injury; Hematopoietic rescue; Pluripotent stem cells; Induced pluripotent stem cells; Mesenchymal stem cells; Very small embryonic-like stem cells; Mobilizing agents

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INTRODUCTION

Accidental radiation exposure and the threat of deliberate radiation exposure have been in the news and are a public health concern. This paper will describe the state of the art of stem cell treatment of acute radiation sickness. Acute radiation sickness is defined as "a combination of clinical syndromes occurring in stages during hours to weeks after exposure as injury to various tissues and organs is expressed"[1]. Experience with acute radiation sickness has been gathered from atomic blast survivors of Hiroshima and Nagasaki and from civilian nuclear accidents as well as experience gained during the development of radiation therapy for cancer. Based on these sources, an approximate dose threshold for each target organ (Table 1) and a time course of illness can be estimated (Table 2).

Note that bone marrow failure (infection, hemorrhage) is not the exclusive cause of death. High dose radiation can kill with cerebral edema and enteritis and pneumonitis, independent of infection. These syndromes are unlikely to be treatable with hematopoietic stem cell transplantation. Pluripotent (ability to differentiate into all three germ layers) stem cells with potential to regenerate multiple tissue types would add an important benefit for the treatment of acute radiation syndrome. Very small embryonic-like stem cells that can be obtained from adults in autologous cell-dose quantities offer such an advantage and are discussed in more detail latter in this paper.

The standard of care is described in the United States Armed Forces Radiobiology Research Institute's "Medical Treatment of Radiological Casualties"[2]. There are many complexities to caring for patients after radiation exposure. Patients who have been exposed to an explosion may have life-threatening injury not related to radiation exposure. Patients may be externally or internally contaminated with radioactive particles. Rapid and effective decontamination can prevent serious sequelæ including bone marrow failure. Another complication is radiation induced emesis which can be dehydrating and limit the utility of orally administered countermeasures. Medical countermeasures for radiation exposure can be classified into 3 groups[3]: (1) Radioprotectants prevent radiation damage to cells (e.g., amifostine); (2) Radiation mitigators limit radiation damage (e.g., pentoxifylline); and (3) Radionuclide eliminators enhance excretion of radionuclides (e.g., Prussian Blue).

The aspects of acute radiation sickness for which hematopoietic stem cell transplantation is appropriate is amelioration of bone marrow suppression and immune suppression and tissue damage repair. This would fit the classification of "radiation mitigators" because it limits damage that has already occurred. For purposes of describing the value of hematopoietic stem cell transplantation in acute radiation sickness, patients who received between 2 Gy -10 Gy are recommended to be treated with white blood cell supporting cytokines, either G-CSF (filgrastim, peg-filgrastim) or GM-CSF (sargramostim). Cytokines are unlikely to be clinically useful in most cases where exposure exceeds 4 Gy. Patients for whom CSFs are unsuccessful are candidates for hematopoietic stem cell transplantation. The published data on the success of bone marrow transplantation following non-therapeutic radiation exposure include the experience of 13 Chernobyl victims described in the next section. In total reports from 58 people exposed to radiation in excess of 5 Gy, half of whom had an allogeneic transplant, revealed that only three of 29 patients transplanted were alive at one year post exposure. Deaths occurred due to the development of graft-*vs*-host disease and other complications unique to allogeneic transplant that could be avoided if autologous bone marrow or blood-derived stem cells were collected and stored before the exposure and used in place of allogeneic cells.

CURRENT INFORMATION ON ACCIDENTAL (CIVILIAN) OR DELIBERATE (MILITARY, TERRORISM) RADIATION EXPOSURE

In addition to approximately 20 civilian and 60 military nuclear accidents, there have been 3 major nuclear accidents as of June 2011: Three Mile Island in the United States, Chernobyl in the former Soviet Union and Fukushima Dai-Ichi in Japan. In these accidents, it is very difficult to quantify the amount of radiation released, but some information is available on acute radiation sickness following the accidents. The Three Mile Island accident during which a portion of the nuclear fuel melted down in a TMI-2 reactor, but did not breach the containment walls, occurred on March 28, 1979. The widespread perception of great danger from this accident was based on expert's concern that the containment vessel might explode, widely distributing radioactive material. In fact, the containment vessel maintained integrity. Even though increased radiation levels were detected inside the plant and at least 50 workers were exposed, no acute radiation sickness from this accident has been reported[4,5].

The Chernobyl accident on April 26, 1986 was much more serious with significant public health consequences. The difficulty in verifying documentation and medical records of the government of the Soviet Union makes an assessment of the extent of acute radiation sickness due to the Chernobyl accident impossible to reconstruct. Nevertheless, a comprehensive review of available information was published by the New York Academy of Sciences in November 2009[6]. The lowest estimate of acute mortality from the Chernobyl disaster is 9000 victims[7]. Soviet physicians reported on 13 bone marrow transplantations for acute radiation sickness due to exposure at Chernobyl. Twelve of 13 patients had skin injuries resembling burns from 20%-100% body surface area in addition to decreasing white blood cell counts. Four of 8 patients with non-HLA identical donors received T-cell depleted bone marrow transplants. Only 2 of the transplant recipients survived to the 3 year follow up. The deaths reported were not attributed to prolonged neutropenia/infection or to thrombocytopenia/bleeding. Interestingly, two of the transplant recipients had evidence of transient engraftment with donor cells followed by recovery of autologous bone marrow[8].

Most recently, on March 11, 2011, a magnitude 9 earthquake followed by a tsunami estimated at 14 meters high, destroyed part of TEPCOs Fukushima Dai-ichi nuclear power plant and resulted in several explosions. The International Atomic Energy Commission Briefing disclosed Fukushima prefecture received 1.5 microSv/h on March 31 over a natural background of 0.1 microSv/h[9]. Two workers were reported to have received radiation burns to ankles when wading in contaminated water[10]. These are the only two cases of acute radiation sickness

Table 1 Approximate threshold doses of conventionally fractionated therapeutic radiation for clinically detrimental nonstochastic effects in various tissues

Organ	Injury at 5 yr	Threshold dose (sv)[1]	Irradiation field (area)
Fetus	Death	2	Whole
Bone marrow	Hypoplasia	2	Whole
Ovary	Permanent sterility	2-3	Whole
Lens	Cataract	5	Whole
Testes	Permanent sterility	5-15	Whole
Cartilage, child	Arrested growth	10	Whole
Breast, child	Hypoplasia	10	5 cm^2
Bone, child	Arrested growth	20	10 cm^2
Bone marrow	Hypoplasia, fibrosis	20	Localized
Muscle, child	Hypoplasia	20-30	Whole
Kidney	Nephrosclerosis	23	Whole
Lymph nodes	Atrophy	33-45	-
Liver	Liver failure, ascites	35	Whole
Lung	Pneumonitis, fibrosis	40	Lobe
Heart	Pericarditis, pancarditis	40	Whole
Stomach, small intestine, colon	Ulcer, perforation	45	100 cm^2
Thyroid	Hypothyroidism	45	Whole
Pituitary	Hypopituitarism	45	Whole
Lymphatics	Sclerosis	50	-
Central nervous system (brain)	Necrosis	50	Whole
Spinal cord	Necrosis, transection	50	5 cm^2
Salivary glands	Xerostomia	50	50 cm^2
Cornea	Keratitis	50	Whole
Capillaries	Telangiectasis, fibrosis	50-60	-
Breast, adult	Atrophy, necrosis	> 50	Whole
Rectum	Ulcer, stricture	55	100 cm^2
Skin	Ulcer, severe fibrosis	55	100 cm^2
Eye	Panophthalmitis, hemorrhage	55	Whole
Oral mucosa	Ulcer, severe fibrosis	60	50 cm^2
Esophagus	Ulcer, stricture	60	75 cm^2
Cartilage, adult	Necrosis	60	Whole
Urinary bladder	Ulcer, contracture	60	Whole
Bone, adult	Necrosis, fracture	60	10 cm^2
Ear (inner)	Deafness	> 60	Whole
Adrenal	Hypoadrenalism	> 60	Whole
Vagina	Ulcer, fistula	90	5 cm
Muscle, adult	Atrophy	> 100	Whole
Uterus	Necrosis, perforation	> 100	Whole

[1]Dose causing effect in 1% to 5% of exposed persons. Modified from[49,50].

reported to date. In October 2011, a consensus document was published that includes additional individual case reports from sparsely documented historical civilian accidental exposures with the caveat that information from those reports was insufficient to guide future therapy[11].

From a perspective on military use of nuclear weapons, the acute radiation sickness due to use of atomic weapons on Hiroshima and Nagasaki during World War Two has been reviewed[12]. survey of 1216 survivors of the blast in Hiroshima, sheltered in a building, revealed that 451 died on the first day and 201 died in the succeeding 2 mo, presumably from the hematopoietic component of acute radiation syndrome. Since transplantation had not been developed, there are no data on bone marrow transplant or stem cell treatment of acute radiation sickness after weapons discharge. It is the mortality figures from the Hiroshima and Nagasaki bombs that form the basis of military mathematical models to predict acute radiation sickness following nuclear weapons discharge.

The United States Health and Human Services' Office of Preparedness and Emergency Operations has made public the scenarios being used to prepare the United States. Two of these scenarios (#1 and #11) include nuclear weapons. These scenarios are being used to plan public health resource prioritizations and can be applied to estimate the number of patients who would potentially benefit from hematopoietic stem cell transplantation. National Planning Scenario #1 envisions a 1 KT nuclear detonation[13]. Col. Jarrett published an estimate of a 4 × 3 km oval that would receive 4 Gy from a 1 kT nuclear detonation[1]. Utilizing published population densities (New York City = 4500 people/km^2 and San Francisco = 5400 people/km^2), this area (9.4 km^2) would represent between 42 000 and 50 000 victims. National Planning Scenario #11 envisions a Radioactivity Dispersal Device ("Dirty Bomb") which would produce a "no entry" zone (> 1 Gy exposure) of 500 m in diameter (0.2 km^2). Utilizing the same published population densities as above, this would represent between 900 and 1080 victims[14]. These estimates demonstrate

Table 2 Symptoms, therapy and prognosis of whole body ionizing radiation injury

	0-1 Sv	1-2 Sv	2-6 Sv	6-10 Sv	10-20 Sv	> 50 Sv
Therapeutic needs	None	Observation	Specific treatment	Possible treatment	Palliative	Palliative
Vomiting	None	5%-50%	> 3 Gy, 100%	100%	100%	100%
Time to nausea, vomiting	-	3 h	2 h	1 h	30 min	< 30 min
Main locus of injury	None	Lymphocytes	Bone marrow	Bone marrow	Small bowel	Brain
Symptoms and signs	-	Moderate leukopenia, epilation	Leukopenia, hemorrhage, epilation	Leukopenia, hemorrhage, epilation	Diarrhea, fever, electrolyte imbalance	Ataxia, coma, convulsions
Critical period	-	-	4-6 wk	4-6 wk	5-14 d	1-4 h
Therapy	Re-assurance	Observation	Transfusion of granulocytes, platelets	Transfusion, antibiotics, bone marrow transplantation	Fluids and salts, possible bone marrow transplantation	Palliative
Prognosis	Excellent	Excellent	Guarded	Guarded	Poor	Hopeless
Lethality	None	None	0%–80%	80%-100%	100%	100%
Time of death	-	-	2 mo	1-2 mo	2 wk	1-2 d
Cause of death	-	-	Infection, hemorrhage	Hemorrhage, infection, pneumonitis	Enteritis, infection	Cerebral edema

Modified from[51].

that even "small" events in a crowded environment may create enormous demands on the local medical system, and would probably exceed the capabilities of almost all facilities.

As discussed earlier, currently available treatment for radiation exposures of greater than 1 Gy are palliative. Hematopoietic stem cell transplantation to rescue patients for whom cytokine therapy failed has several limitations. The primary limitation is that the donor pool is limited by the need for at least partial HLA matching. As an example, the United States National Marrow Donor Program reports among 9 million donors, only 650 000 (7%) are African American, making bone marrow matching for African Americans difficult[15]. Similar problems probably exist for other under-represented ethnic groups. Once the hematopoietic transplant has engrafted, there is continuous need for immunosuppression. In addition to the risks of life-threatening infection during titration of immunosuppressant medication, some of these medications have dose limiting acute and chronic toxicity independent of graft-*vs*-host disease[16].

Autologous hematopoietic stem cell treatment would solve the problems of immunosuppression and graft-*vs*-host disease. If people at risk were to receive G-CSF mobilized cells collected prior to exposure than there would be sufficient cells available to prevent the profound cytopenia and immune suppression that follows exposure to 4 Gy or more of radiation. In addition, a small volume of bone marrow (100-200 mL) collected prior to exposure and then expanded *ex vivo* post exposure, may also be sufficient to reconstitute hematopoiesis and immune function. The major concern is whether hematopoietic stem cells capable of re-constituting the bone marrow could be expanded *ex vivo* from 100-200 mL, before bone marrow suppression became life-threatening. Clinical studies using marrow, mobilized blood and cord blood have demonstrated the feasibility of doing so[17-19]. Harvesting hematopoietic stem cells from damaged marrow is being done with complex protocols in cancer treatment. However, there are many reports of protocols failing to mobilize hematopoietic stem cells sufficient for reconstitutive use. For example, fludarabine exposure in adults with follicular lymphoma predicted a poor hematopoietic stem cell harvest evidenced by > 5 d apheresis requirement[20]. In a retrospective analysis of 204 patients, Ford *et al*[21] calculated that platinum based drugs and etoposide exposure were most highly correlated with poor hematopoietic stem cell mobilization as reflected by the absence of CD34+ cells on the first day that the white blood cell count was greater than 500. Stem cell mobilization was reported successful in only 12 of 20 (60%) patients with chronic lymphocytic leukemia[22]. These results suggest that chemotherapy treatment at a minimum impairs hematopoietic stem cell mobilization. In addition, a new study confirms expectations, that age between 65-69 years impairs hematopoietic stem cell mobilization relative to younger patients with the same disease[23]. In contrast, hematopoietic stem cell harvest in children is not limited by mobilization, but by scaling factors in extracorporeal volumes and anticoagulation necessary for the apheresis machine and vascular access for sufficient flow[24]. No reports of hematopoietic stem cell harvest from pregnant women could be found on PubMed search. Ford *et al*[21] did not find any correlation of poor hematopoietic stem cell mobilization with prior radiotherapy which gives these authors hope that victims of acute radiation sickness could have their hematopoietic stem cells successfully harvested. However, radiation damage may result in long term issues such as myelodysplasia and leukemia so use of cells previously stored and not exposed to radiation would appear optimal.

NEW PROCEDURES ON THE HORIZON

New mobilizing agents

New mobilizing agents are being developed to replace the colony stimulating factors. The hematopoietic stem

cell harvesting described above utilized G-CSF (filgrastim or peg-filgrastim) and/or GM-CSF (sargramostim) as mobilizing agents. The newer agent, Mozobil® (perixaflor) was approved in the United States in 2009 and acts by reversibly binding CXCR4 and inhibiting CXCR4/CXCL12 anchoring[25]. Mobilization with Mozobil® (plerixaflor) increased successful 4 d apheresis harvesting from 88% (136/154 patients) with G-CSF (filgrastim)alone to 95% (141/148 patients) with both G-CSF (filgrastim) and Mozobil® (plerixaflor)[25]. Natalizumab is an antibody in development as a mobilizing agent that binds to VCAM-1 and interferes with VCAM-1/VLA-4 anchoring[26].

Though un-glycosylated thrombopoietin combined with G-CSF was effective at mobilizing hematopoietic stem cells, the risk of developing autoimmune thrombocytopenia led to the cessation of development of unglycosylated thrombopoietin[27]. The effect of thrombopoietin agonists on animal models of radiation induced thrombopenia are in progress for the peptide Nplate (romiplostim) and orally dosed small molecule Promecta (eltrombopag) and full length, glycosylated, recombinant human thrombopoietin[27].

Burdelya *et al*[28] reported mouse radio-protective activity from a *Salmonella enterica* flagellin derivative given 1 h after radiation exposure and rhesus monkey protection when given 45 min prior to radiation exposure. Its putative mechanism of action is *via* toll like receptor 5 (TLR5) to nuclear factor-κB (NF-κB) signaling to multiple cytokines including G-CSF. This product is under active development by Cleveland Biolabs, Inc., (Buffalo, NY).

Parathyroid hormone (PTH) appears to mobilize stem cells to peripheral blood in mice with a distinct mechanism from G-CSF[26]. In a Phase 1 study in patients with at least one failed peripheral stem cell harvest attempt, the combination of PTH (teraparitide) for days 1-14 and G-CSF (filgrastim) for days 10-14 prior to apheresis resulted in 9/20 (45%) patients meeting pre-specified mobilization criteria. The authors note that this level of success was also seen in second mobilization attempts with a combination of filgrastim (G-CSF) and sargramostim (GM-CSF). A spontaneous observation study of patients with primary hyperparathyroidism showed CD45+/CD34+/c-kit+ and CD45+/CD34+/CXCR4+ bone marrow progenitor cells were increased relative to matched controls. Interestingly, the primary hyperparathyroidism patients had lower G-CSF dosing than controls, while stem cell factor and erythropoietin were not different between groups[29]. Another spontaneous observation study evaluated hemodialysis patients with varying levels of secondary hypo- and hyper-parathyroidism. In patients with high PTH, circulating hematopoietic stem cells were higher than controls or patients with normal PTH levels. In patients with low PTH, circulating stem cell numbers were lower than patients with normal or elevated PTH[30].

Lastly, α-tocopherol succinate is being explored as a single dose inducer of endogenous G-CSF equivalent to a multiday course of G-CSF (filgrastim)[31].

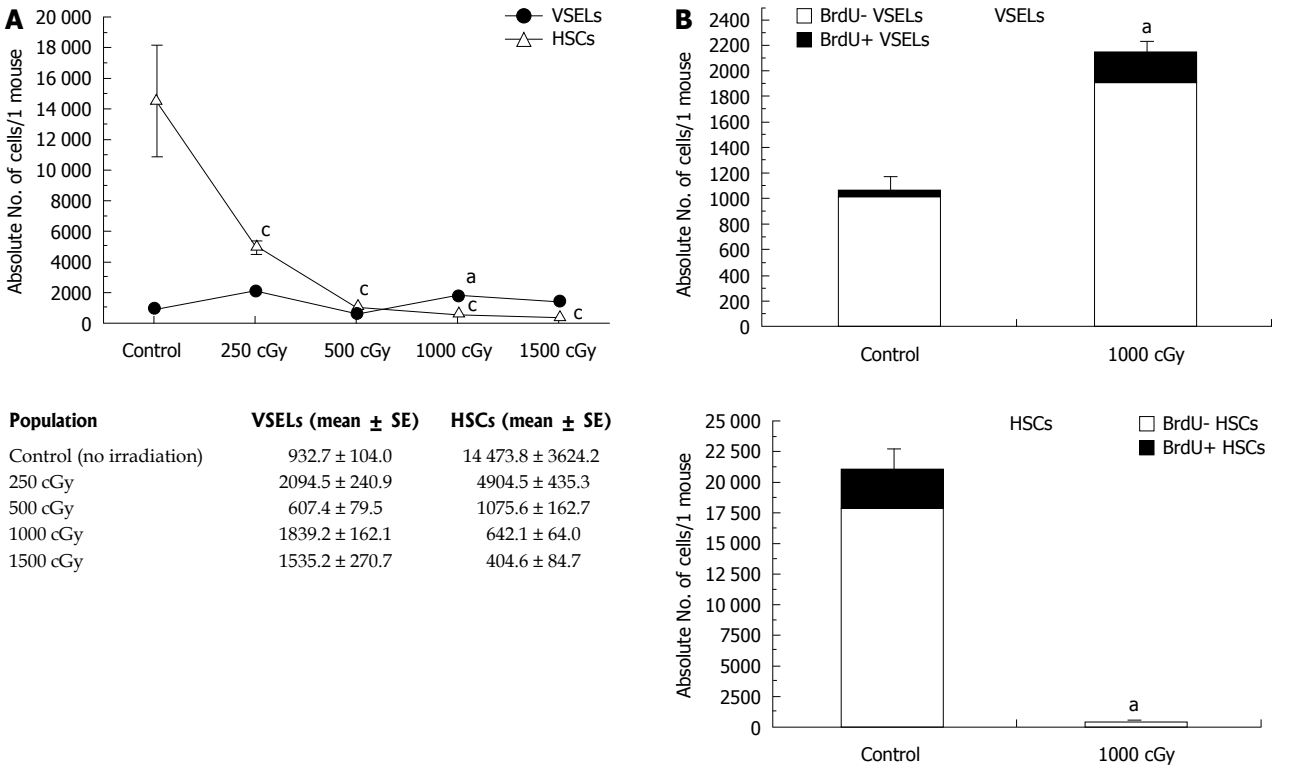
STEM CELL THERAPY

Induced pluripotent stem cells

Another source of cells to reconstitute radiation damaged bone marrow would be adult induced pluripotent stem cells (iPS). Recent laboratory work shows that fibroblasts can be induced to become pluripotent stem cells without using retroviruses, only using mRNA for key transforming factors[32]. Human iPS maintain a flat colony morphology in the laboratory when maintained with basic Fibroblast Growth Factor. Induction of iPS into hematopoietic stem cells covering all three lineages (myelopoietic, erythropoietic and thrombopoietic) has not yet been described. Current laboratory confirmation of pluripotency is the ability of iPS to form teratomas *in vitro* and *in vivo*. Though this is a large potential clinical problem, progress continues to be made and a recent report describes reprogramming skin fibroblasts from a patient with thalassemia (single gene mutation)[33]. Laboratory size colonies appeared in 3 wk following transfection with an engineered retrovirus and were induced to form "hemaglobinized" (HbF producing) colonies in another 2 wk using specially prepared growth media. No description of resulting myeloid or thrombopoietic cells were offered[33]. In addition, the "retro-differentiation" process currently requires approximately 100 adult cells to create 1 induced pluripotent stem cell (1% efficient) and the cells so produced exhibit early senescence[34]. Recently Zhao *et al*[35] have shown that iPS can be rejected due to abnormal gene expression in some iPS cells which can induce a T-cell- dependent immune response. These authors recommend that the immunogenicity of autologous cells should be carefully evaluated before these cells are considered for therapeutic purposes. So as of this writing, iPS are not on the near-horizon for clinical use in acute radiation sickness. Barriers that will need to be overcome to use iPS to treat acute radiation sickness include: production of all three bone marrow lineages from iPS; managing the risk of using oncogene sequences for induction or the identification of alternate induction techniques; improving the speed of hematopoietic stem cell production to a timeframe consistent with rescuing the patient from bone marrow failure; eliminating the risk of rejection; and scaling up production for mass casualties.

Mesenchymal stem cells

A commercial product of human mesenchymal stem cells (MSC) prepared from multiple bone marrow donors (Prochymal®) is being developed for acute Graft-*vs*-Host Disease in bone marrow transplants. Since one lineage of MSC matures into bone marrow stromal cells, MSC have been considered as a supportive treatment for primary engrafting of bone marrow transplants. The initial publication in 2007 that human MSC home to radiation-damaged tissue in mice provided evidence of the potential for restorative therapy outside of the bone marrow[36]. Hu *et al*[37] demonstrated that MSC rescued



Population	VSELs (mean ± SE)	HSCs (mean ± SE)
Control (no irradiation)	932.7 ± 104.0	14 473.8 ± 3624.2
250 cGy	2094.5 ± 240.9	4904.5 ± 435.3
500 cGy	607.4 ± 79.5	1075.6 ± 162.7
1000 cGy	1839.2 ± 162.1	642.1 ± 64.0
1500 cGy	1535.2 ± 270.7	404.6 ± 84.7

Figure 1 Resistance of very small embryonic-like stem cells to γ-radiation. The content of very small embryonic-like stem cells (VSELs) and HSCs was evaluated in murine BM following whole-body irradiation with different doses of g-radiation (250, 500, 1000 and 1500 cGy) when compared to control (no irradiation). A: Absolute numbers of VSELs (Sca-1+/Lin-/CD45-) and HSCs (Sca1+/Lin-/CD45+) in BM after 4 d post-irradiation. The table presents mean numbers of VSELs and HSCs per mouse (mean ± SE). $^{a}P < 0.05$ *vs* control (VSELs); $^{c}P < 0.05$ *vs* control (HSCs); B: Absolute numbers of VSELs and HSCs incorporating BrdU following whole-body irradiation. Data are presented as mean absolute numbers of VSELs and HSCs per mouse (mean ± SE) (From Ref.[43], with permission). $^{a}P < 0.05$ *vs* Control.

fatally irradiated mice. Lange *et al*[38] extended the findings to show that MSCs effect rescue in fatally irradiated mice by anti-inflammatory and "hematopoietic stem cell niche modulating" effects such that endogenous hematopoiesis recovers. Thus, it is possible that MSCs may indeed have a beneficial effect in acute radiation sickness, however it is credible that this effect is due to paracrine, trophic and endogenous stem cell recruitment, rather than a regenerative capability. As the work of Heider *et al*[39] would suggest, bone marrow derived pluripotent stem cells give rise to MSCs and thus are supportive of the role of MSCs in hematopoiesis, but MSCs themselves are not regenerative.

In this setting, Osiris Therapeutics and Genzyme Corporation are collaborating to develop Prochymal® for the treatment of acute radiation sickness. Current clinical trials in steroid refractory graft-*vs*-host disease, where patients have received significant radiation exposure to treat their underlying disease, will provide a human model on which to base animal studies of Acute radiation sickness[40].

Myeloid progenitor cell product

Though not a stem cell product, CLT008, being developed by Cellerant Therapeutics (San Carlos, CA), is a multi-person sourced human cell population derived from donor bone marrow. This product is reported to have capability to mature into monocytes, neutrophils and red blood cells. Though it does not have the potential to mature into T and B lymphocytes, it is being considered as a temporary therapy until the host marrow recovers[41].

Very small embryonic-like cells

Very small embryonic-like cells (VSELs) are pluripotent and present in many tissues and circulate in peripheral blood. The properties and therapeutic potentials of VSELs have been recently reviewed[42]. VSELs can differentiate into multiple cell types *in vitro* and in mice[43]. Kassmer *et al*[44] recently reported that bone marrow derived stem cells that were not hematopoietic were able to differentiate into type 2 pneumocytes in fatally irradiated mice. These small bone marrow cells were reported to be identical to VSELs (personal communication, DS Krause, 2010). VSELs have been documented to be present during routine bone marrow or hematopoietic stem cell harvesting[45]. Ratajczak and colleagues report that in addition to hematopoietic stem cells, VSELs are mobilized during burn injury[46]. This adds important information to the mobilization of VSELs during acute myocardial infarct and stroke in humans[47,48]. So it is likely that some spontaneous mobilization of VSELs will be happening during acute radiation sickness. Murine VSELs are highly radiation-resistant relative to a general population of hematopoietic stem cells, tolerating 1 Gy of γ radiation

and retaining *ex vivo* pluripotent differentiating activity (Figure 1)[43]. Also important is that the *ex vivo* expansion of VSELs requires only 5-10 d in culture[43]. Barriers that will need to be overcome to use VSELs to treat acute radiation sickness include: confirming radio-resistant characteristics of VSELs in humans; confirming that VSEL expansion using growth media doesn't activate oncogenes; and scaling up production for mass casualties.

CONCLUSION

Bone Marrow reconstitution as a partial treatment for acute radiation sickness has developed significantly since bone marrow transplantation was utilized for Chernobyl disaster victims in 1986. Use of autologous bone marrow or mobilized and harvested hematopoietic stem cells should eliminate the risk of graft-*vs*-host disease. The potential of autologous sourced stem cells is being evaluated now. Autologous cell sources include induced hematopoietic stem cells, induced pluripotent stem cells from adult differentiated tissue, MSC from bone marrow, myeloid progenitor cells from bone marrow, and VSEL stem cells from peripheral blood. Autologous human VSELs are emerging as fully functional stem cells that not only have wide-ranging regenerative competence, but have the critically important attribute of radiation resistance. The ultimate goal will be utilizing autologous, expanded stem cell infusions that would reconstitute many of the tissues damaged by radiation exposure.

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REVIEW

Natural compounds as anticancer agents: Experimental evidence

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Abstract

Cancer prevention research has drawn much attention worldwide. It is believed that some types of cancer can be prevented by following a healthy life style. Cancer chemoprevention by either natural or synthetic agents is a promising route towards lowering cancer incidence. In recent years, the concept of cancer chemoprevention has evolved greatly. Experimental studies in animal models demonstrate that the reversal or suppression of premalignant lesions by chemopreventive agents is achievable. Natural occurring agents such as dietary phytochemicals, tea polyphenols and resveratrol show chemopreventive activity in animal models. Moreover, clinical trials for testing the safety and efficacy of a variety of natural agents in preventing or treating human malignancy have been ongoing. Here, we summarize experimental data on the chemopreventive or tumor suppressive effects of several natural compounds including curcumin, (-)-epigallocatechin-3-gallate, resveratrol, indole-3-carbinol, and vitamin D.

Key words: Cancer; Chemoprevention; Natural agents; (-)-Epigallocatechin-3-gallate; Resveratrol; Curcumin; Vitamin D; Indole-3-carbinol

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INTRODUCTION

Cancer is common disease that limits lifespan. Many factors including life style, genetic variation, virus infection and chronic inflammation may affect the susceptibility to cancer. In the past decades, both the diagnosis and treatment of malignant tumors are improving. In addition to traditional treatments such as chemotherapy and radiotherapy, molecular targeted therapy is emerging as a promising trend for cancer therapeutics. For those who are at high risk for cancer, chemoprevention may be an alternative intervention to inhibit or delay carcinogenesis. While a number of chemotherapeutic agents have been administered in the clinic for many years, there is still long way to go for chemopreventive agents to be safely and effectively administered in human populations. The identification of chemopreventive targets and biomarkers that can help monitoring their effectiveness are a huge challenge. In addition to synthetic compounds, many natural products have been found to be able to inhibit carcinogenesis, at least in animal models. There are many ongoing clinical trails to test the safety and efficacy of natural agents in preventing or treating cancer (Table 1). It is highly likely that natural agents can be used for cancer prevention

without recognizable adverse effects. Here, we highlight the experimental evidence concerning some natural agents that exhibit protective effect against cancer. The sources of some natural compounds are shown in Figure 1.

CURCUMIN

Curcumin, a polyphenolic molecule isolated from the roots (rhizomes) of the plant Curcuma longa, is a promising compound for cancer chemoprevention and therapy[1,2]. Although Curcuma longa and its chemical components have been used in Chinese and Hindu medicine for thousands of years, curcumin has attracted much attention in recent decades because of its anticancer activity. The beneficial effects of curcumin include anti-oxidant, anti-inflammatory, anti-proliferative, and anti-angiogenic properties[1,3]. Many preclinical studies of curcumin have shown anti-carcinogenic and therapeutic effects in various tumor cell lines and xenograft models. The anticancer efficacy of curcumin is well established in a range of animal cancer models including those associated with colon, breast, pancreas, lung, kidney, bladder, blood and skin[1]. It is worth mentioning that curcumin has the ability to kill cancer cells selectively without apparent toxicity to nonmalignant cells, a good property for a cancer-preventive candidate[4]. Extensive toxicological screening and preclinical investigation showed minimal adverse effects of curcumin administration in mice, rats, dogs, and monkeys[5]. Phase I and phase Ⅱ clinical trials have already demonstrated the safety of curcumin even at high doses (8-12 g/d) over several months. Adverse events were mainly nausea and diarrhea[6-9].

Regardless of its excellent safety profile, the poor solubility and low bioavailability of curcumin are obstacles to therapeutic drug development. Data on the pharmacokinetics, metabolites, and systemic bioavailability of curcumin in rodents and humans show that curcumin is poorly absorbed, rapidly metabolized, and may have limited systemic bioavailability[10,11]. In fact, after oral administration of curcumin, very low concentrations of curcumin or corresponding metabolites are found in patient serum and tissues outside the gastrointestinal tract[8,12,13]. Compared to the effective concentrations *in vitro* (5-50 μmol/L), the poor absorption and bioavailability of curcumin suggest that its anticancer effects may be limited *in vivo*. Some approaches to improving the bioavailability of curcumin have been investigated, including the combination with adjuvants, the use of chemical analogues and novel delivery methods[14,15].

It is interesting that studies in animals still show curcumin is an effective agent for several cancer models, in spite of its limited bioavailability. It is not clear whether this efficacy comes from unmeasured curcumin metabolites, or for other unknown indirect effects. The pharmacodynamic data in humans is still limited. Currently, several phase I and phase Ⅱ clinical trials are ongoing to investigate the benefits of curcumin as a chemopreventive and chemotherapeutic agent in a variety of cancers, including multiple myeloma, pancreatic cancer, breast cancer and colon cancer (www.clinicaltrials.gov). According to reported data from these ongoing studies, some results are promising. Results from a phase I clinical study of twenty-five patients with various pre-malignant or high-risk lesions suggested that oral curcumin may have chemopreventive effects on these lesions (histological improvement)[8]. In another study from the Cleveland Clinic in Florida, five patients with familial adenomatous polyposis were treated with a combination of curcumin and quercetin three times a day for a mean duration of 6 mo. The numbers and sizes of polyps were reduced in all patients compared to baseline values[16]. A recent phase Ⅱ clinical trial reported by Carroll and colleagues investigated curcumin's potential activities for prevention of colorectal neoplasia in smokers with aberrant crypt foci (ACF). The data showed a significant reduction of ACF number by curcumin at the 4-g dose level and indicated that curcumin may have cancer-preventive effects in the setting of early pre-invasive neoplastic lesions. Interpretation of this study, however, was limited by the remaining controversy around ACF as a biomarker of colon carcinogenesis, nonrandomization and lack of placebo group[17].

Curcumin efficacy in the treatment of human pancreatic cancer has been reported in a phase Ⅱ clinical trial in patients with advanced disease. Patients received 8 g curcumin by mouth daily until disease progression, with restaging every 2 mo. Twenty-one of twenty-five patients were evaluable for response. Low concentrations of curcumin were able to elicit a biological effect by down-regulating the expression of nuclear factor κB (NF-κB), cyclooxygenase (COX)-2 and phosphorylated signal transducer and activator of transcription 3 in peripheral blood mononuclear cells derived from patients[9]. A further phase Ⅱ clinical studies suggested that a combination of gemcitabine and curcumin is a feasible treatment for patients with pancreatic cancer[18]. Bayet-Robert *et al*[19] treated 14 advanced and metastatic breast cancer patients with a combination of curcumin and docetaxel. The study demonstrated that the combination therapy decreased the vascular endothelial grow factor (VEGF) levels and showed encouraging efficacy. Hopefully, there will soon be more data to demonstrate the anticancer effects of curcumin, especially measurements which confirm the mechanism-based molecular targets which are really implicated *in vivo*.

(-)-EPIGALLOCATECHIN-3-GALLATE

Tea, from the plant Camellia sinensis, is one of the most popular beverages consumed worldwide. The most abundant chemical compound in green tea is catechins, which include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocalechin, (-)-epicatechin-3-gallate and (-)-epicatechin. Among these, EGCG accounts for more than 50% of the total catechins, and appears to be the most effective and best-studied constituent of green tea[20].

EGCG holds considerable promise for chemoprevention according to epidemiological, cell culture, animal

Table 1 Selected ongoing clinical trials with natural anti-cancer compounds

Agent	Phase	Status	Trial type	Conditions/cancer type	Combinati-on	Trial No.
Curcumin	Phase Ⅱ	Recruiting	Prevention	Familial adenomatous polyposis		NCT00641147
	Phase Ⅰ	Completed	Prevention	Healthy		NCT00027495
	Phase Ⅰ Phase Ⅱ	Recruiting	Treatment	Advanced osteosarcoma		NCT00689195
	Phase Ⅱ	Recruiting	Treatment	Advanced pancreatic cancer		NCT00094445
Tea polyphenols	Phase Ⅱ	Recruiting	Prevention	Postmenopausal women with high breast density		NCT00917735
	Phase Ⅱ	Recruiting	Prevention	Tobacco use disorder		NCT00611650
	Phase Ⅰ	Recruiting	Prevention	Premalignant lesions of the head and neck	Erlotinib	NCT01116336
	Phase Ⅰ	Recruiting	Treatment	Small cell lung carcinoma		NCT01317953
	Phase Ⅱ	Recruiting	Treatment	Multiple myeloma and plasma cell neoplasm		NCT00942422
	Phase Ⅱ	Completed	Treatment	Bladder cancer		NCT00088946
Indole-3-carbinol/ 3,3-diindoly-lmethane	Phase Ⅱ Phase Ⅲ	Completed	Prevention	Healthy men at risk for prostate cancer progression		NCT00579332
	Phase Ⅰ	Recruiting	Prevention	Women with a BRCA1 mutation		NCT01022333
	Phase Ⅰ Phase Ⅱ	Completed	Treatment	Prostate cancer	Radical prostatectomy	NCT00450229
	Phase Ⅱ Phase Ⅲ	Recruiting	Treatment	Breast cancer		NCT01391689
Vitamin D		Completed	Observation of the relationship between vitamin D level and thyroid cancer	Thyroid cancer		NCT00719615
	Phase Ⅱ	Recruiting	Prevention	Postmenopausal women at high risk for breast cancer		NCT00859651
	Phase Ⅲ	Enrolling by invitation	Prevention	Adenomatous colon polyps	Calcium	NCT00399607
	Phase Ⅰ Phase Ⅱ	Recruiting	Treatment	Metastatic melanoma	Temozolomide	NCT00301067
	Phase Ⅱ	Completed	Treatment	Metastatic or locally advanced pancreatic cancer	Docetaxel	NCT00238199
	Phase Ⅳ	Completed	Prevention of osteoporosis	Postmenopausal breast cancer survivors	Risedronate; calcium	NCT00567606

Data from the United States National Institutes of Health, http://www.clinicaltrials.gov/.

and clinical studies. EGCG has been shown to cause growth inhibition and apoptosis in a number of human cancer cell lines *in vitro* and inhibit tumor incidence and multiplicity in animal models, such as liver, colon, prostate, pancreas, mammary glands, lung and skin cancer models[21]. The mechanisms underlying EGCG anticancer effects include anti-oxidant activities, modification of carcinogen metabolism, prevention of DNA damage, induction of cell cycle arrest and apoptosis, inhibition of metastasis, proteasome inhibition and modulation of multiple signal transduction pathways [epidermal growth factor receptor, human epidermal growth factor receptor 2, VEGF receptor, insulin-like growth factor (IGF)1R, phosphoinositide 3-kinase/AKT, mitogen-activated protein kinase and NF-κB signaling][22]. Abundant evidence from animal cancer models has demonstrated the strong chemopreventive effects of EGCG. As early as 1987, Yoshizawa *et al*[23] reported that the application of EGCG suppressed 7,12-dimethylbenz[a]anthracene (DMBA) plus teleocidin-initiated carcinogenesis. EGCG significantly decreased tumor incidence and burden per mouse compared with controls. The subsequent studies showed EGCG to have a broad spectrum against carcinogens. In the animal model, EGCG effectively inhibited (4-methylnitro-samino)-1-(3-pyridyl)-1-butanon (NNK) and benzo(a)pyrene-induced lung cancer, azoxymethane-induced ACF and colon tumors, NNK and diethylnitrosamine-induced liver tumors, UV-induced skin cancer, and N-butyl-N-(4-hydroxybuty1)-nitrosamine-induced urinary bladder tumors[24].

Despite the impressing anti-tumor effect of EGCG in animal, the available epidemiological evidence on tea consumption and cancer prevention in humans has not yielded conclusive results. The inconsistent results of epidemiological studies were probably due to various confounding factors. The quantity and quality of the tea consumed will definitely affect the outcome of epidemiological studies. In addition, the effect of caffeine in tea, large intersubject and intrasubject variability could be additional contributing factors to the inconsistency[24]. Furthermore, the concentrations of EGCG in plasma and tissue after simply drinking green tea is low compared to the effective concentrations used in cell culture experiments (10-100 μmol/L)[20]. To minimalize the confounding effects, more potent tools were used in later well-designed clinical intervention studies, including a defined green tea catechin (GTC) extract, EGCG-enriched fractions such as Polyphenone E (the EGCG content about

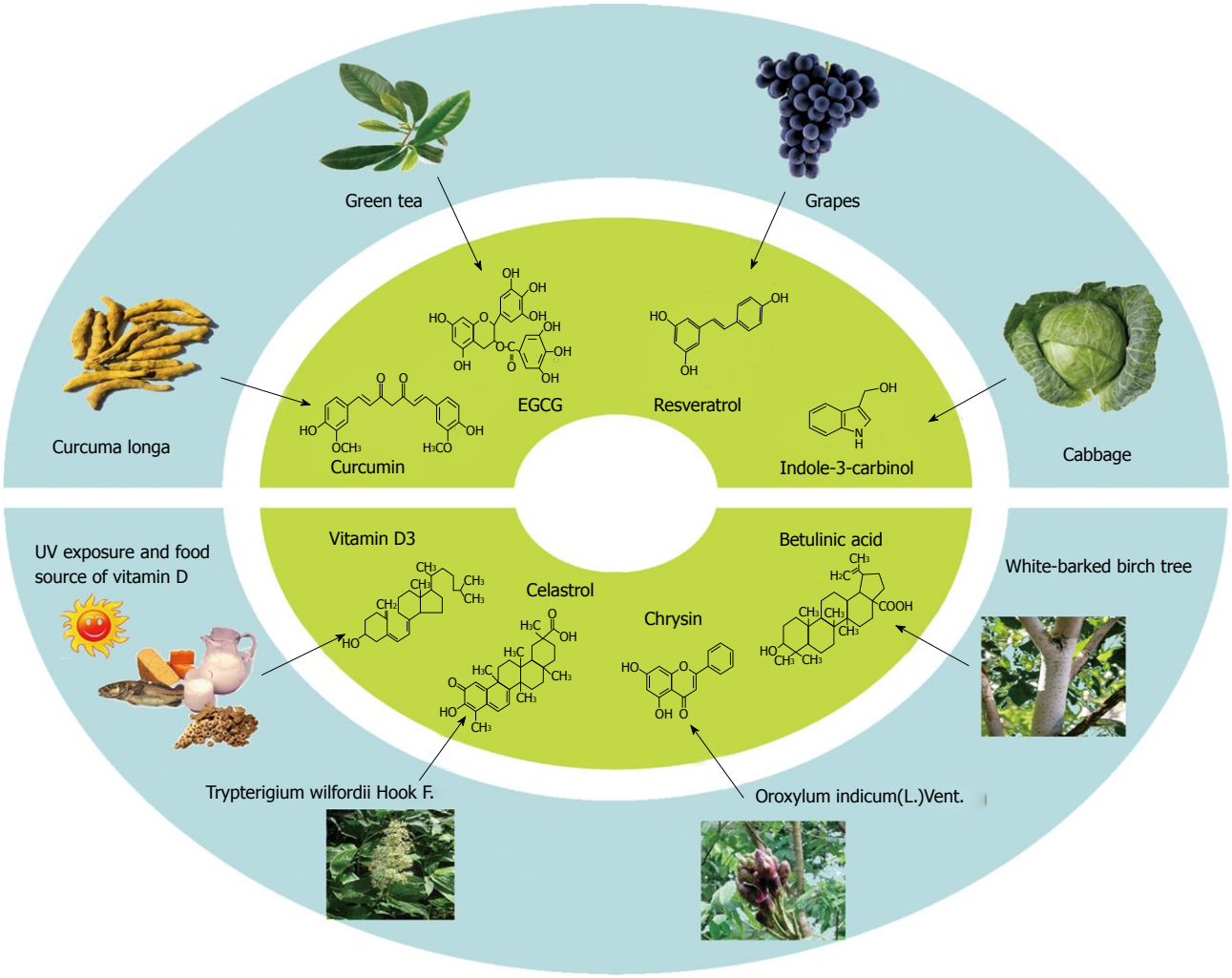

Figure 1 The sources of natural anti-cancer agents, including curcumin, (-)-epigallocatechin-3-gallate, resveratrol, indole-3-carbinol, and vitamin D, chrysin, celastrol and betulinic acid.

70%) and highly purified EGCG provided by a pharmaceutical company.

Several studies of the systemic bioavailability of orally administered catechins in human volunteers have been conducted. Chow *et al*[25,26] conducted several studies to examine the safety, tolerability, and pharmacokinetic properties of single and multiple dose administration of EGCG and Polyphenon E from 200 to 800 mg. Their studies showed that the oral bioavailability of tea polyphenols in humans was low. Oral administration of EGCG and Polyphenon E at the same dose level (based on EGCG content) resulted in similar plasma EGCG levels. However, the repeated administration of 800 mg of green tea polyphenols once daily for 4 wk resulted in a 60% increase in the systemic availability of free EGCG, which may be due to inhibition of presystemic elimination of this catechin. The majority of clinical studies demonstrate the safety and limited side effects of EGCG. However, a recent review suggested a causal association between green tea and liver damage. This hepatotoxicity may be attributed to EGCG or its metabolites which, under particular conditions related to the patient's metabolism, can induce oxidative stress in the liver[27].

Some data are currently available from EGCG chemoprevetion and chemotherapy trials, which offer us more details of EGCG action in the human body. Ahn and coworkers reported that oral treatment of polyphenon E or purified EGCG, 200 mg daily for 12 wk, was effective in patients with human papilloma virus (HPV)-infected cervical lesions[28]. A pilot study conducted in Japan, investigated the effect of green tea extract (GTE) on metachronous colorectal adenomas. Oral administration of GTE, 1.5 g/d for 12 mo, in addition to a tea drinking life-style, showed efficacy in preventing the incidence of metachronous adenoma in patients 1 year postpolypectomy. The incidence of metachronous colorectal adenomas at end-point colonoscopy was 31% (20 of 65) in the control group and 15% (9 of 60) in the GTE group[29]. Another encouraging clinical trial investigated possible prostate cancer chemoprevention with oral GTCs. Sixty volunteers with high-grade prostate intraepithelial neoplasia received either 600 mg of GTCs or placebo daily for 12 mo. The primary end point was prevalence of prostate cancer. After 1 year of follow-up, only 3% of

the patients in the treatment group developed prostate cancer, compared with 30% in the placebo group[30]. In a 2-year follow-up, despite the high drop-out rate (57% in the placebo-arm and 55% in the GTCs-arm), three further cancer diagnoses appeared. One prostate cancer was diagnosed among 13 GTC-treated patients and 2 among 9 placebo-treated patients. Overall, treatment with GTCs led to almost 80% reduction in prostate cancer diagnosis. These results suggest that the inhibition of prostate cancer progression after 1 year of GTCs administration was long-lasting and no adverse effect was associated with the treatment[31].

The results from clinical trials with EGCG are not all positive. A phase I study performed in 49 patients with various tumours reported no major antitumor responses when using GTE at the maximum-tolerated dose of 4.2 g/m^2 once daily or 1.0 g/m^2 three times day[32]. In a Phase Ⅱ study, green tea showed minimal anti-neoplastic activity, as defined by a decline in prostate specific antigen (PSA) levels, among 42 patients with androgen independent prostate carcinoma. Only a single patient manifested a 50% decrease in PSA level from baseline and this response was not sustained beyond 2 mo. Green tea toxicity occurred in 69% of patients and included nausea, emesis, insomnia, fatigue, diarrhea, abdominal pain, and confusion[33]. On the other hand, other therapeutic trials with EGCG have shown promising results. A recent phase Ⅱ clinical trial demonstrated the effects of short-term supplementation with Polyphenon E on serum biomarkers in prostate cancer patients. Twenty-six men were given daily doses of Polyphenon E (total of 800 mg EGCG) before radical prostatectomy (average drug administration of 6 wk). Polyphenon E administration significantly reduced serum levels of hepatocyte growth factor, VEGF, PSA, IGF-I, IGF binding protein (IGFBP)-3, and the IGF-I/IGFBP-3 ratio with no adverse effects on liver function. These findings support a potential role for Polyphenon E in the treatment or prevention of prostate cancer[34]. Shanafelt *et al*[35] reported that EGCG induced apoptotic cell death in the leukemic B-cells from a majority of patients with chronic lymphocytic leukemia (CLL), and four patients with low grade B-cell malignancies developed positive responses shortly after self-initiating EGCG therapy by oral ingestion of EGCG containing products. Based on this evidence, the same group conducted a phase I trial to define the clinical benefit of Polyphenon E. Thirty-three previously untreated patients with asymptomatic Rai stage 0 to Ⅱ CLL received Polyphenon E treatment. Declines in absolute lymphocyte count (ALC) and/or lymphadenopathy were observed in the majority of patients. One patient achieved partial remission and more than 50% of study patients attained a sustained decline in ALC of 20% and a 50% reduction in lymphadenopathy at some point during treatment. No differences in response were observed based on IgVH, ZAP-70, or CD38 gene mutation status except for trisomy 12[36]. Furthermore, this research group is conducting an ongoing phase Ⅱ trial to evaluate efficacy of Polyphenon E at 2000 mg dose, twice a day in patients with asymptomatic Rai stage 0 to Ⅱ CLL (www.clinicaltrials.gov).

While EGCG alone is active in suppressing cancer, combination of EGCG with other agents may be more promising. EGCG reportedly exhibits synergistic effects with other anti-cancer drugs, such as curcumin, chrysin, tamoxifen, etoposide, 5-fluorouracil, temozolomide, taxane, erlotinib[37-43]. Consequently, several clinical trials of EGCG in combination with other drugs for cancer treatment are now ongoing. On the other hand, recent studies indicate that EGCG may be able to block the therapeutic efficacy of some anticancer agents such as bortezomib and other boronic acid-based proteasome inhibitors. This should be highly relevant for clinical considerations[44,45].

RESVERATROL

Resveratrol, a polyphenol, was first isolated in 1940 as an ingredient of the roots of white hellebore (Veratrum grandiflorum O.Loes) and has since been found in various food sources including red wine, grapes, mulberries, peanuts[46,47]. Resveratrol was identified in 1963 as the active constituent of the roots of Polygonum cuspidatum, a plant used in Chinese and Japanese traditional medicine[46]. Jang *et al*[48] reported the ability of resveratrol to inhibit carcinogenesis at multiple stages, including initiation, promotion and progression. Subsequent studies demonstrated the strong chemopreventive efficacy of resveratrol in many different animal models of carcinogenesis. Oral or local application of resveratrol in mice or rats significantly reduced DMBA-initiated and 12-O-tetradecanoylphorbol-13-acetate-promoted skin tumors, suppressed DMBA-induced mammary carcinogenesis, inhibited 1,2-dimethylhydrazine-induced colon carcinogenesis and N-nitrosomethylbenzylamine-induced esophageal tumors. Overall, the majority of these studies strongly support the chemopreventive effect of resveratrol, although there are exceptions in which a lack of *in vivo* benefit has been observed[49-53].

Besides its chemopreventive effects, extensive study over the past decade has suggested that resveratrol might be a promising candidate for cancer therapy by interfering with many signaling pathways that regulate cell proliferation, apoptosis, inflammation, angiogenesis and metastasis[46,47]. It suppresses the proliferation in a wide variety of human tumor cells *in vitro* and in xenograft models. Resveratrol was also reported to exhibit synergistic chemopreventive effects with other anti-cancer drugs, such as cisplatin, doxorubicin and vinorelbine[54-56]. However, a recent study indicated that resveratrol can significantly attenuate the efficacy of paclitaxel's anticancer actions in certain human breast cancer cell lines both *in vitro* and *in vivo*[57], suggesting that concomitant use of resveratrol with paclitaxel may be detrimental in certain types of human cancers. More preclinical and clinical testing of the potential benefits and risks of using resveratrol as an anticancer adjuvant in cancer patients is warranted.

The bioavailability and the pharmacokinetics of resveratrol have been studied in experimental animals and humans. These studies showed that resveratrol was rapidly absorbed after oral intake, and rapidly metabolized to glucuronide and sulphate conjugates which result in the low concentrations of resveratrol observed in plasma[47]. The low bioavailability led to uncertainty over whether oral resveratrol can reach the bioactive concentrations in target tissues. The limited data from research about the tissue distribution of resveratrol and its metabolites offers us some clues. Data from mice demonstrated the significant accumulation of resveratrol in the intestine, stomach, liver, kidney and bile[58,59]. In a clinical study, a level of resvertrol was found in colon tissue in excess of that required for activity *in vitro*, which supported the colon as a target organ for oral resveratrol in humans[60].

A wide range of doses of resveratrol (0.1-1500 mg/kg) was used in animal studies with various effecicacy and low toxicity, although more human studies are needed to establish the relevant dose for human use. To date, limited data was obtained from human studies performed with resveratrol, and it is difficult to compare the results concerning the safety and tolerability of resveratrol because of variations in conditions of administration (e.g., pure resveratrol formulation, or other non-pure resveratrol samples in various matrices). It was generally agreed by the expert panel of Resverarol 2010 that at least some portion of the population is likely take 1-2 mg of resveratrol per day from dietary sources and at this amount is almost certain to be safe for chronic consumption[61].

Two clinical studies in healthy volunteers investigated the cancer-preventive effect of resveratrol through examination of related biomarkers. A phase I study carried out in forty healthy volunteers showed that ingesting a range of doses of resveratrol (0.5, 1.0, 2.5 or 5.0 g daily) for 29 d caused a decrease in circulating IGF-1 and IGFBP-3, respectively, compared to pre-dosing values. At the 2.5 g dose level, the decrease was most marked. The observed decrease in circulating IGF-1 and IGFBP-3 may contribute to cancer chemopreventive activity. Several other potential markers of activity were also investigated in blood samples from the volunteers. Resveratrol neither significantly affected circulating levels of prostaglandin E-2 (PGE-2), reflecting perturbation of the arachidonic acid cascade, nor influenced leukocyte levels of the malondialdehyde-DNA adduct M1dG, reflecting DNA oxidation[62]. Consistent with the evidence *in vitro* and in animal models, another clinical study performed in healthy volunteers showed that resveratrol intervention inhibited the phenotypic indices of CYP3A4, CYP2D6, and CYP2C9 and induced the phenotypic index of 1A2. In addition, in individuals with low baseline GST-π levels and UGT1A1 activity, intervention was associated with a significant increase in enzyme activity. Modulation of enzyme systems involved in carcinogen activation and detoxification could be one of the mechanisms responsible for the cancer preventive effect of resveratrol. However, such activities may also alter the pharmacokinetics of other drugs. Therefore, the authors suggested that further clinical studies should consider evaluation of lower doses of resveratrol to minimize adverse metabolic drug interactions[63].

Based on the evidence from animal studies which showed oral administration of resveratrol can efficiently induced apoptosis in colon cancer with high levels achievable in local tissue, most of the clinical therapeutic trials have focused on investigating the effects of resveratrol on colon cancer. The first reported clinical trial of resveratrol in patients with colon cancer was conducted to assess the effects of a low dose of a plant-derived resveratrol formulation and resveratrol-containing freeze-dried grape powder (GP) on biomarkers related to the Wnt pathway, a key signaling pathway activated in over 85% of colon cancers[64]. Eight patients received 14 d of treatment until the day prior to surgery for colon cancer resection. Resveratrol and GP had significant activity in inhibiting Wnt targets on normal colonic mucosa, such as cyclin D1 and axin Ⅱ. However, GP treatment increased the expression of some Wnt target genes in colon cancer, including myc and cyclin D1. Resveratrol may have more clinical utility for colon cancer prevention rather than for treatment of established colon cancer[64]. In another clinical study, twenty patients with histologically confirmed colorectal cancer consumed 8 daily doses of resveratrol at 0.5 or 1.0 g prior to surgical resection. Resveratrol was found to be well tolerated. With respect to its activity in target tissues, resveratrol slightly inhibited cell proliferation in colorectal cancer tissue after ingestion, as assessed by Ki-67 immunostaining[60]. A recent report of a phase I , randomised, double-blind clinical trial, described the effects of SRT501 (micronized resveratrol) in patients with colorectal cancer and hepatic metastases[65]. Cleaved caspase-3, a marker of apoptosis, was significantly increased by 39% in malignant hepatic tissue following SRT501 treatment, compared to tissue from placebo-treated patients. However, SRT501 failed to change the levels of several other biomarkers associated with cell survival and apoptosis in plasma or in tumor tissues, including PGE-2, VEGF, IGF-1, Ki67, phosphor-Akt (ser473), Akt1, phospho-GSK3, GSK3, phospho-extracellular signal-regulated kinase (ERK), ERK, phospho-JNK, JNK, β-catenin, survivin, BCL2, Bax or PARP[65].

Cellular senescence is an anticancer mechanism that our organism may implement to arrest cancer cells. The arrest of senescence and inhibition of cancer growth appear to be two antagonistic activities. However, it is well documented that resveratrol has both anti-senescence and anti-cancer activities, indicating that it has complex roles in preventing ageing and carcinogenesis. In addition, the numerous formulations of resveratrol used in clinical research and its potential interactions with other drugs make it difficult to recommend an optimal dosage for clinical usage. Long-term clinical trials are needed to validate the anti-cancer effect of resveratrol when used as a drug or as food supplement.

INDOLE-3-CARBINOL

Indole-3-carbinol (I3C), an indole compound, is naturally found in many plants, particularly in cruciferous vegetables such as broccoli, cabbage, cauliflower, Brussels sprouts, and bok choy[66,67]. Glucobrassicin, a major component of cruciferous vegetables, is hydrolyzed in acidic conditions to give I3C[68]. I3C is chemically unstable in aqueous and gastric acidic environments, such as those encountered under cell culture conditions and the acidic environment of the stomach *in vivo*. In acidic conditions, I3C is rapidly converted to numerous condensation products, of which 3,3-diindolylmethane (DIM) is the most active and effective metabolite[66,69]. The effect of I3C *in vivo* might, at least in part, be attributable to the formation of DIM.

To date, I3C and its metabolite, DIM, have been demonstrated in numerous epidemiological and preclinical studies to possess cancer preventive properties. *In vitro* studies demonstrated that both I3C and DIM inhibit growth of most types of hormone-dependent and -independent cancer cells (breast, prostate, liver, lung, colon, cervix, and ovarian cancers)[66,70-72]. In addition, in *in vivo* studies, I3C and DIM have been shown to have pronounced chemopreventive effects against growth of both spontaneous and chemically induced cancers in various animal models[73-77]. The anti-cancer properties of I3C is attributable to its ability to modulate multiple signaling pathways which control DNA repair, hormonal regulation, inflammation, cell division and growth, apoptosis, angiogenesis, and multiple drug resistance[66,78,79]. I3C has been to shown to induce phase 1 and phase 2 enzymes that metabolize carcinogens, prevent carcinogen-DNA adduct formation, regulate several nuclear transcription factors [such as estrogen receptor (ER), aryl hydrocarbon receptors (AhR, Sp1 and NF-κB)], modulate anti-apoptotic and pro-apoptotic factors, repress extracellular matrix-degrading proteases, and reverse the process of epithelial mesenchymal transition *via* regulation of key miRNAs[66,67,80-82]. Among these multiple mechanisms, the most important effect of I3C and DIM is modulation of estrogen metabolism. I3C and DIM has received special attention as an effective chemopreventive agent against hormone-dependent cancers such as breast, cervical and prostate cancers, for the most part, due to its ability to negatively regulate ERα signaling and alter cytochrome P450-mediated estrogen metabolism[83,84]. Despite the low affinity of I3C for ERα, it significantly inhibits ERα activity thereby diminishing estrogen-mediated cellular and biochemical effects in estrogen-responsive cells and tissues[85]. I3C could also induce ER protein ubiquitination and degradation in a process requiring AhR, which binds to a wide range of ligands including DIM[86,87]. In addition, a recent study has identified DIM as a ligand-independent activator of ERβ, a molecule associated with antiproliferative activity in breast cancer cells[88]. Moreover, I3C and DIM may reverse the metabolism of estradiol to a more beneficial pathway, thus reducing levels of toxic 16α-OHE_1 and increasing levels of protective 2-OHE_1, which correlates with reduced risk of breast cancer and other cancers including cervical and prostate cancer[89].

Several studies have been conducted to detect the pharmacokinetics of I3C. Upon oral administration of 250 mg/kg I3C to female CD-1 mice, I3C and its acid condensation products were absorbed and distributed systemically into a number of well-perfused tissues[90]. In contrast, in human testing, no I3C was found in the plasma after giving a single dose of up to 1200 mg or multiple-doses at 400 mg administered twice daily for 4 wk, and DIM was the only detectable I3C-derived compound in plasma[91]. These results support the concept that I3C may serve as the prodrug rather than the actual therapeutic agent. Most clinical data on I3C and DIM indicate a good safety profile and only minor adverse effects. Rosen *et al*[92] reported a long term clinical study using I3C for the treatment of recurrent respiratory papillomatosis. Among 11 patients having a complete response to I3C, the average number of months on I3C was 50.2 mo, and no immediate or long-term side effects were found. However, I3C also has been found to promote cancer of the liver in rats, raising some doubt its use[93,94].

Both I3C and DIM have already undergone human clinical trials, most of them focused on investigating effects on hormone responsive cancers, including cervical dysplasia, breast cancer, vulvar intraepithelial neoplasia (VIN), and prostate cancer. Similar to the effects observed in animal models, several studies showed that I3C and DIM strongly affect estradiol metabolism in healthy humans[95,96]. They were able to induce the activity of cytochrome P450 isoenzyme CYP1A2, and increase the ratio of 2-OHE_1:16α-OHE_1. A similar beneficial shift in estrogen metabolites was also observed in early studies on women with increased risk for breast cancer[97-99] and a more detailed study on postmenopausal women with a history of early-stage breast cancer[100]. Based on the preclinical evidence which indicated that I3C and DIM may offer benefit for diseases caused by HPV, two studies explored the effect on cervical intraepithelial neoplasia (CIN). Bell and colleagues used I3C administered orally to treat women for CIN. Thirty patients with biopsy-proven CIN Ⅱ-Ⅲ were randomized to receive placebo or 200 or 400 mg/d I3C for 12 wk. There was a statistically significant regression of CIN in patients treated with oral I3C compared with placebo. In addition, the 2/16α-hydroxyestrone ratio changed in a dose-dependent fashion[101]. In another randomized clinical trial, oral DIM at a dose of 2 mg/kg per day for 12 wk was well tolerated with no significant toxicity and clinically significant improvement was demonstrated in patients with grade 2 or 3 CIN[102]. Naik *et al*[103] reported the results of a randomized phase Ⅱ trial of I3C in the treatment of VIN. In this study, 12 women were randomized to receive 200 or 400 mg/d of I3C, following histological confirmation of high-grade VIN. I3C administration led to a significant reduction in symptoms, lesion size, and severity as well as significant improvement in estrogen metabolism.

Recently, in a phase I clinical trial, Rajoria *et al*[104] recommended DIM as an anti-estrogenic dietary supplement to help reduce the risk of developing thyroid proliferative disease based on the fact that DIM was detected in thyroid tissue and that DIM supplementation significantly improved estrogen metabolism.

VITAMIN D

In addition to its originally identified role on calcium homeostasis and bone metabolism, vitamin D is being recognized as a steroid hormone which exerts a wide range of biological activities related to various clinical disorders including cancer[105,106]. Vitamin D_2 is mainly produced by the irradiation of yeast or plant ergosterol[106]. In humans, vitamin D_3 (cholecalciferol) is synthesized naturally in skin cell by exposing to ultraviolet B radiation in sunlight, the major source of vitamin D for most humans. In the skin, 7-dehydrocholesterol, a cholesterol precursor is converted to vitamin D_3. Vitamin D_3 is hydroxylated to 25-hydroxyvitamin D_3 (25-OH-D_3) in the liver by 27-hydroxylase and further converted to 1α,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$,calcitriol) by 1α-hydroxylase in the kidney and other tissues. Calcitriol is mainly catabolized by 24-hydroxylase (CYP24A1) to 1α,24,25-$(OH)_2D_3$, removing its bioactivity[107].

Most of the anticancer effects of 1,25-$(OH)_2D_3$ are mediated through binding to its specific receptor, the vitamin D receptor (VDR). In the cell nucleus, 1,25-$(OH)_2D_3$ binds to VDR, which subsequently heterodimerizes with another nuclear receptor, the retinoid X receptor[106,108]. The heterodimer binds to vitamin D responsive element in target genes and initiates the regulation of specific genes, including those involved in the regulation of cell growth, differentiation, apoptosis, and inflammation, the key mechanisms underlying the development and progression of cancer[109-112]. 1,25-$(OH)_2D_3$ has been shown to have significant anticancer effect on prostate, colon, breast, lung, liver, skin and pancreatic cancer cells, which express VDR[105,112]. In addition, through a so-called non-genomic mechanism, 1,25-$(OH)_2D_3$ may have rapid effects on cellular functions[113,114].

Substantial experimental studies, *in vitro* and in animals, showed the significant antitumor action of vitamin D and thoroughly investigated mechanisms at cellular and molecular levels[115-117]. Furthermore, a large number of epidemiological studies explored the relationships between cancer incidence and geographic location, ultraviolet irradiation, and circulating levels of 25-OH-D_3[118-125]. Despite abundant experimental evidence in support of an inverse association between vitamin D status and cancer risk, the available epidemiological evidence has not demonstrated consistently positive results to date. With some exceptions, most epidemiological studies have reported that populations in areas with low UV exposure have an increased risk of various types of cancers such as prostate, colon and breast[119-125].

Serum 25-OH-D_3 level is the most widely used indicator of vitamin D status in relation to other vitamin D metabolites[126]. The result of a large population-based case-control study supported by the Long Island Breast Cancer Study Project showed that plasma 25-OH-D_3 level was inversely associated with breast cancer risk in a concentration-dependent fashion. Women with circulating 25-OH-D_3 above 40 ng/mL had approximately a 40% reduction in breast cancer risk compared with those who were vitamin D deficient[127]. A large population-based case-control study from Germany reported similar findings[128]. However, results from prospective studies did not support an association between vitamin D status and breast cancer. The inconsistent results also extend to studies of other types of cancer[129]. A recent meta-analysis suggested that, in well-fed populations, an inverse relationship between serum 25-OH-D_3 levels and colorectal cancer existed (2630 cases in 9 studies), while no association was found for breast (6175 cases in 10 studies) and prostate cancer (3956 cases in 11 studies)[130].

Excessive vitamin D intake was associated with additional toxic effects, such as hyperphosphatemia and hypercalciuria with clinical symptoms including nausea and vomiting, dehydration, muscle weakness, lethargy and confusion. The toxicity generally occurred when the daily dose exceeds 10 000 IU of vitamin D on a chronic basis[131,132]. In this respect, several vitamin D analogs were synthesized with an attempt to minimize these side effects[117,133]. In addition, recent investigations have followed the approach of intermittent administration of calcitriol in very high doses which elicits its antiproliferative effects with only transient hypercalcemia[134,135]. There are still many unresolved questions regarding the maximum tolerated dose, optimal biologic dose and the optimal schedule for available vitamin D formulations.

A number of cancer intervention trials in humans have been conducted using vitamin D and its metabolites, as well as analogs alone or in combination with other anticancer drugs for the prevention or treatment of various cancers, especially prostate cancer, breast cancer and colorectal cancer. Similar to the results of the epidemiological studies, the outcomes of the clinical trials are inconclusive. The largest number of clinical studies have attempted to assess the efficacy of vitamin D in prostate cancer patients. Several phase Ⅱ studies with calcitriol in combination with various chemotherapies showed a decrease in prostate-specific antigen levels. Additionally, in a large randomized, double-blinded, phase Ⅱ trial (called ASCENT I) in patients with advanced prostate cancer (n = 250), the administration of a high dose (45 mg) of calcitriol (DN101) in combination with docetaxel caused a significant improvement in overall survival while there were no changes in the PSA response. Unfortunately, the 900 patient phase Ⅲ study (ASCENT Ⅱ) was stopped early because of inferior survival in the DN101 group, thus failing to confirm the improved survival seen in the ASCENT I trial[136]. In a recent phase Ⅱ study, high-dose intravenous calcitriol at a dose of 74 μg weekly in combination with dexamethasone failed to produce a

clinical or PSA response in men with advanced prostate cancer[137]. As regards breast cancer, the large intervention trial in 36 282 postmenopausal women conducted by the Women's Health Initiative showed calcium plus vitamin D supplementation for an average of 7 years did not reduce the incidence of invasive breast cancer compared with placebo[138]. In contrast, another randomized study of vitamin D supplementation (cholecalciferol) indicated that diagnosis of all invasive cancers including breast cancer and colon cancer was substantially reduced in the vitamin D replacement group[139]. For colorectal cancer, Fedirko *et al*[140] conducted a double-blind, 2 × 2 factorial clinical trial to test the effects of calcium and vitamin D_3 alone or in combination on markers of apoptosis in the normal colorectal mucosa. Bax expression along the full length of the crypts increased by 56% in the vitamin D group (*vs* placebo). However, another randomized trial indicated that calcium with vitamin D supplementation had no detectable effect on the incidence of colorectal cancer[141].

OTHER PROMISING NATURAL AGENTS

Besides the natural agents mentioned above, there are other natural compounds with chemopreventive and chemotherapeutic potential. Chrysin, a natural flavonoid found in many plants, honey and propolis, possesses strong anti-inflammatory and anti-oxidant activity, and exhibits anti-cancer activity against leukemia, malignant glioma, breast carcinoma, cervical cancer, prostate cancer, lung cancer and colon cancer[142]. Chrysin may inhibit cell proliferation and induce apoptosis by cell cycle arrest, inactivation of Akt signaling, activation of caspases, suppression of COX-2 and NF-κB activation, and inhibition of proteasome activity[142,143]. Chrysin has also been shown to reverse multidrug resistance of cancer cells *via* inhibition of P-glycoprotein and breast cancer resistance protein (BCRP/ABCG2)[144,145]. However, there are few clinical study of the activity of chrysin against human cancers. Celastrol, a quinone methide triterpene, is the major active compound derived from the root of Tripterigium wilfordii Hook F. (the Chinese Thunder of God Vine), and generally used for the treatment of inflammatory and auto-immune diseases[146]. Celastrol has attracted considerable attention recently, for its potential anti-cancer effects, with a broad spectrum of activity against multiple cancer types in both cell culture and animal models. Several molecular mechanisms of celastrol have been identified, including inhibition of IKK-NF-κB signaling, disruption of the Cdc37/Hsp90 interaction, proteasome inhibition and heat shock response activation[147,148]. However, no systematic clinical trials in human subjects have been carried out with celastrol to date. Betulinic acid is a natural product that is present in a variety of plants, especially the white-barked birch tree. The molecule is a member of the triterpene family of compounds that exhibit a variety of biological activities, especially, potent anti-HIV-1 and antitumor properties[149,150]. This triterpene has been found to inhibit the proliferation of a variety tumor cells and suppress tumor growth in animal studies. Numerous molecular targets for betulinic acid have been reported including enzymes (kinases, aminopeptidase N, acetyl-CoA acetyltransferase, topoisomerase Ⅰ/Ⅱ), the proteasome, NF-κB and cell cycle regulation[150,151]. In future studies, more anticancer mechanisms of betulinic acid will be delineated and clinical studies are needed to confirm the therapeutic value and exact anticancer target of betulinic acid in the human body.

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BRIEF ARTICLE

Indirect calorimetry in obese female subjects: Factors influencing the resting metabolic rate

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Abstract

AIM: To evaluate selected factors influencing resting energy expenditure (REE) in obese female subjects.

METHODS: Seventy seven 61 obese Caucasian women [mean age of 52.93 ± 13.45 years, and mean body mass index (BMI) of 41.78 ± 11.54 kg/m^2] were enrolled; measurements of resting metabolic rate (RMR) by a ventilated, open-circuit system, indirect calorimeter were performed after an overnight fast. Body composition as well as medications, physical parameters, blood samples, disease pattern, and smoking were considered.

RESULTS: RMR was significantly associated with body weight (r = 0.732, P < 0.001), body height (r = 0.401, P = 0.008), BMI (r = 0.504, P < 0.001), waist circumference (r = 0.602, P < 0.001), mid-upper arm circumference (r = 0.417, P = 0.006), mid-upper arm muscle circumference (r = 0.344, P = 0.028), total body water (r = 0.339, P = 0.035), body temperature (r = 0.409, P = 0.007), smoking (P = 0.031), serum T_4 levels (r = 0.331, P = 0.036), obstructive sleep apnoea syndrome (OSAS; P = 0.023), impaired glucose tolerance (IGT; P = 0.017) and impaired glycaemic status, including hyperinsulinism, IGT and diabetes mellitus (P = 0.003).

CONCLUSION: Future research should be prompted to optimize the procedure of indirect calorimetry to achieve clinical benefits in obese subjects.

Key words: Indirect calorimetry; Obesity; Resting metabolic rate; Resting energy expenditure

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INTRODUCTION

An imbalance between energy expenditure (EE) and energy intake (EI) underlies the accumulation of exceeding body fat (BF) in obese subjects[1,2]. Moreover, adiposity represents a common soil at the origin of different disorders such as type 2 diabetes mellitus (T2DM), hyperten-

sion, dyslipidemia, coronary heart disease (CHD), metabolic syndrome (MetS), sleep apnoea, as well as cancer[3].

In overweight and obese patients, the exact assessment of basal metabolism plays a pivotal role in order to tailor a balanced nutritional support. Predictive equations have been originally developed from data collected in normal-weight individuals[4,5]; even if they are considered to be a rapid and easy indirect method of resting metabolic rate (RMR) definition, they could not be thoroughly appropriate for obese patients, as a lack of correspondence between predicted values and real metabolic rate has been described in this subset of subjects[6].

The direct measurement of RMR should be performed when the clear- cut definition of energy requirements is needed to address correctly dietary interventions[7]. Indirect calorimetry (IC) is based on the evaluation of O_2 consumption (Vo_2) and CO_2 production (Vco_2), and it is the most common method currently used with this purpose[8].

Several factors have been shown to influence RMR measurement by IC. FFM, gender, and age may affect RMR, whereas the role of other factors, such as T2DM, ethnicity, menstrual cycle, hypertension, thyroid function, and smoking, remains to be univocally clarified[9,10].

The aim of this study was the analysis of selected factors potentially affecting the RMR for a better understanding of determinants of RMR, providing evidence that would be helpful in obesity management and prevention of obesity-related comorbidities, optimizing weight loss strategies.

MATERIALS AND METHODS

Subjects

Sixty-one obese Caucasian women (mean age: 52.93 ± 13.45 years, and mean BMI: 41.78 ± 11.54 kg/m^2), were enrolled in the study. Secondary diseases and medications at the time of the recruitment were not considered as exclusion criteria. After the IC performance, 19 women were excluded because the steady state criterion was not achieved. Hence, a sample of 42 female obese subjects was considered.

Study design

Examinations were performed under thermo-neutral conditions, from 8 to 10 o'clock in the morning[11]. Participants had to remain in a 12-h fasting state before the examination, refraining from any heavy physical activity in the same period, abstaining from smoking in the preceding 2 h, and without ingesting coffee or water during 4 h prior to examination[12].

RMR measurement

RMR was defined for each subject using an open- circuit indirect computerized calorimeter (stable IC: Quark RMR Cosmed), equipped with a canopy. Measurements were performed along a 15-min period preceded by a 10-min rest. Thermo-neutral conditions in a darkened room were chosen, leaving subjects in a comfortable environment. Subjects were asked not to move and not to talk during the test performance. Heart rate (HR) was monitored. Excluding the preliminary 5 min of each measurement, the subsequent 10 min were considered for the evaluation of data, expressed in kJ/d; also the steady state criterion was established (5-min stable period of < 10% variation in measured VO_2 and VCO_2). O_2 consumption and CO_2 production were standardized for temperature, barometric pressure and humidity[13].

Anthropometric parameters

Body weight (BW) was measured to an accuracy of 0.1 kg through a standard column body scale. Body height (BH) was determined using a rigid stadiometer to an accuracy of 0.5 cm. BMI was calculated as BW/(BH)^2. Waist circumference (WC) was gathered using a standard measuring tape, to an accuracy of 0.1 cm. Mid arm circumference (MAC) was gathered using a standard measuring tape, on the non-dominant arm midway between the shoulder and elbow, to an accuracy of 0.1 cm.

Body composition

Bioimpedance analysis was performed on the right body side[14] using a bioimpedance analyser AKERN Bioresearch SRL, Pontassieve, FL, Italy. Skinfold measurements: fat mass (FM) was estimated from the sum of four skinfolds (SF): tricipital (TSF), bicipital, subscapular, and suprailiac, measured on the non-dominant body side. Each measurement was repeated for three times, and the mean value was calculated to reduce the variability in the performance (Harpenden Caliper User manual). Body density (BD) was assessed using the Durnin and Womersley sex- and age- adjusted linear regression equation; from BD, FM was calculated by the Siri equation[15].

Physical parameters

Measurements of blood pressure (BP) were performed on the left upper arm, to an accuracy of 5 mmHg. The body temperature (BT) was measured in the armpit, using a standard clinical thermometer.

Laboratory data/ biochemistry

Blood samples were taken from each subject after an overnight fast. The following biochemical parameters were assayed: glucose, insulin, triglycerides (TG), total cholesterol (TC), LDL-cholesterol, HDL-cholesterol, thyroid stimulating hormone (TSH), thyroxin (T_4), albumin, and pre- albumin.

Laboratory tests were performed using a COBAS-MIRA analyser and a Cell-Dyn 1700 Analyser (Abbott), at the Laboratory of the "Villa delle Querce" Clinical Rehabilitation Institute. Serum concentrations of the biological indices were determined by routine methods with conventional commercial kits (ABX, Rome, Italy).

Disorders, diseases, medications and lifestyle habits

These parameters encompassed: T2DM (defined as fast-

Table 1 Anthropometric characteristics, clinical parametersand body composition

Variable	mean ± SD
Age (yr)	52.72 ± 13.96
BW[1] (kg)	102.97 ± 26.55
BH[1] (cm)	158.41 ± 6.71
BMI[1] (kg/m^2)	41.35 ± 11.37
WC[1] (cm)	115.44 ± 22.08
HR[1] (bpm)	66.61 ± 11.03
SBP[1] (mmHg)	127.32 ± 18.06
DBP[1] (mmHg)	78.04 ± 8.58
CB[1] (cm)	39.01 ± 8.23
CMB[1] (cm)	29.31 ± 5.27
FM[1,2] (%)	42.86 ± 10.06
FM[1,3] (%)	45.34 ± 28.07
FFM[1,3] (%)	54.66 ± 28.07
MM[1,3] (%)	32.16 ± 25.71
BCM[1,3] (%)	44.49 ± 8.40
BCM[1]-Index[3]	8.91 ± 2.43
TBW[1,3] (%)	41.06 ± 20.42
ECW[1,3] (%)	52.56 ± 5.37
ICW[1,3] (%)	47.44 ± 5.37
RMR[1] (kcal/d)	1537.91 ± 281.76

Descriptive data in obese women. [1]See the full text for abbreviations of subjects' anthropometric parameters; [2]Estimated by Harpenden caliper; [3]Estimated by BIA. BW: Body weight; BH: Body height; BMI: Body mass index; WC: Waist circumference; HR: Heart rate; FM: Fat mass; RMR: Resting metabolic rate.

ing glucose levels ⩾ 126 mg/dL), impaired glucose tolerance (IGT) (fasting glucose levels ranging from 110 to 126 mg/dL), hyperinsulinism, obstructive sleep apnoea syndrome (OSAS), hypothyroidism/dysthyroidism, dyslipidemia, hypertension (BP > 130/85 mmHg), depression. Metformin, levothyroxine, anti- depressants and antihypertensive agents were included. Smoking habit (more than ten cigarettes per day), and the menstrual state (regular/irregular menses and postmenopausal period) were also taken into account.

Statistical analysis

Baseline data and subjects' characteristics were evaluated and collected. Statistical analysis was performed using Excel 2007 and SPSS 10.0 for Windows. Parameters were expressed by mean values and by standard deviation (SD) indicating the minimal and maximal extremity of each range of values. The outcome dependent variable, the RMR, was expressed in kcal/d. Independent variables were expressed as continuous or as cathegorial variables.

Pearson's correlation coefficient was used to correlate the means of the RMR with parameters. Linear regression models, analysis of variance (ANOVA), and independent *t* test were used. Differences and correlations were considered statistically significant when *P* value was < 0.05.

RESULTS

A total of sixty-one women (mean age of 52.93 ± 13.45 years, and mean BMI of 41.78 ± 11.54 kg/m^2), were studied. Descriptive data, anthropometric characteristics, and selected parameters are shown in Tables 1 and 2, respectively. Just forty two of the sixty one women achieved the steady state criterion. Thus, for further evaluation, 42 obese women (mean age 52.72 ± 13.96 years and mean BMI 41.35 ± 11.37 kg/m^2) were considered only.

Table 2 Parameters influencing resting metabolic rate (descriptive data)

Variable	Subjects (%)
T2DM	33.3
IGT	18.8
Hyperinsulinism	26.1
OSAS	17.4
Dysthyroidism	31.3
Dyslipidemia	13.0
Hypertension	65.2
Depression	27.5
Metformin	36.2
Antidepressants	18.8
Antihypertensive drugs	10.1
Levothyroxine	31.9
Smoking habit	43.8
Postmenopausal state	0

T2DM: Type 2 diabetes mellitus; IGT: Impaired glucose tolerance; OSAS: Obstructive sleep apnoea syndrome.

Table 3 Correlation analysis among subjects' anthropometric parameters, body composition and resting metabolic rate (kJ/d) (n = 42)

Variables	Correlation (r)	P[4]
Age (yr)	0.006	0.969
BW[1] (kg)	0.732	< 0.001
BH[1] (cm)	0.401	0.008
BMI[1] (kg/m^2)	0.504	< 0.001
WC[1] (cm)	0.602	< 0.001
MAC[1] (cm)	0.417	0.006
MAMC[1] (cm)	0.344	0.028
FM[1,2] and FFM[1,2] (%)	0.169	0.290
FM[1,3] and FFM[1,3] (%)	0.113	0.485
MM[1,3] (%)	0.105	0.522
BCM[1,3] (%)	0.033	0.837
BCM[1]-Index[3]	0.120	0.458
TBW[1,3] (%)	0.339	0.035
ECW[1,3] and ICW[1,3] (%)	0.029	0.865

[1]See the full text for abbreviations of subjects' anthropometric parameters; [2]Estimated by Harpenden caliper; [3]Estimated by BIA. BW: Body weight; [4]Differences and correlations were considered statistically significant when *P* value was < 0.05. BH: Body height; BMI: Body mass index; WC: Waist circumference; FM: Fat mass.

Factors influencing the RMR

Anthropometric parameters influencing the RMR: The following anthropometric parameters: BW (r = 0.732, P < 0.001), BMI (r = 0.504, P < 0.001), WC (r = 0.602, P < 0.001), MAC (r = 0.417, P = 0.006), BH (r = 0.401, P = 0.008), MAMC (r = 0.344, P = 0.028), and TBW (r = 0.339, P = 0.035), showed a statistically significant correlation with RMR (Table 3).

Table 4 Correlation of subjects' physical characteristics with resting metabolic rate (n = 42)

Variables	Correlation (r)	P[1]
Blood pressure (mmHg) systolic/diastolic	0.048/0.035	0.759/0.825
Heart rate (b/min)	0.296	0.050
SpO_2 (%)	0.158	0.318
Body temperature (℃)	0.409	0.007

[1]Differences and correlations were considered statistically significant when P value was < 0.05.

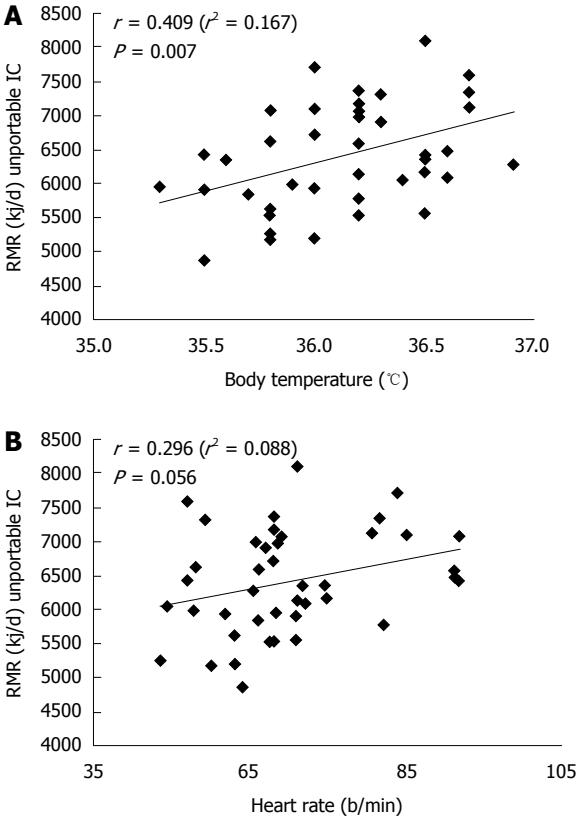

Figure 1 Correlation analysis between resting metabolic rate and body temperature (A) and heart rate (B). RMR: Resting metabolic rate.

Physical parameters influencing the RMR: BP, HR, SpO_2, and BT were considered (Table 4). No significant correlation was found for BP (r = 0.048/0.035, for systolic and diastolic BP, respectively) and SpO_2 (r = 0.158) indicating a lack of influence of these parameters on the measured RMR. 29 (69%) of the total subjects were treated with anti- hypertensive agents, likely accounting for normal BP values. On the other hand, correlations were obtained with HR (r = 0.296, P = 0.050; Figure 1B: P < 0.05), and a significant association was found with BT (r = 0.409, P = 0.007; Figure 1A). As shown in Figure 1A (P < 0.05), BT was positively correlated with RMR.

A slight positive correlation between RMR and HR was described, showing a trend toward a higher estimated RMR consistently with an increase of HR, but without statistically significant results.

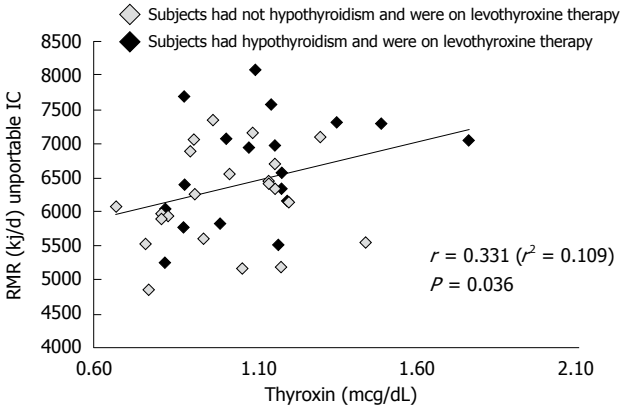

Figure 2 Correlation analysis between resting metabolic rate and thyroxin levels. RMR: Resting metabolic rate.

Blood parameters influencing the RMR: In Table 3 correlation analysis among RMR and serum TC, TG, LDL- cholesterol, HDL- cholesterol, glucose, insulin, TSH, T_4, albumin and pre-albumin levels is shown. Just one significant positive association has been reported between RMR and serum T_4 concentrations (r = 0.331, P = 0.036) (Figure 2: P < 0.05); as illustrated in Figure 2, a significant positive correlation has been found between RMR and T_4 concentrations, even considering progressively increasing serum T_4 levels within the normal range of values. Eighteen subjects had hypothyroidism and were on levothyroxine therapy (Figure 2).

Other factors influencing the RMR: A correlation analysis among RMR and disorders, diseases, medications, and smoking was performed. A total of 13 parameters were included: type 2 diabetes mellitus (T2DM- including patients on metformin therapy), IGT, hyperinsulinism, thyroid function (hypothyroidism and dysthyroidism), dyslipidemia, hypertension (including subjects on hypertensive therapy), OSAS, depression, anxiety, menstrual cycle or postmenopausal state, antidepressant medications, metformin, levothyroxine, and smoking. A significant relationship was demonstrated between RMR and smoking (P = 0.031), OSAS (P = 0,023), IGT (P = 0.005), and impaired glycemic status- including IGT, T2DM, and hyperinsulinism (P = 0.003).

Environmental conditions influencing the RMR: Data provided by correlation analysis excluded any significant association among RMR and environmental variables (room temperature: r = 0.118, P = 0.456; room humidity: r = 0.076, P = 0.634, and atmospheric air pressure: r = 0.018, P = 0.910).

DISCUSSION

In the present study we selected and considered anthropometric and physical characteristics, blood parameters, as well as environmental conditions, to ascertain their impact on the RMR of obese female subjects.

As expected, BW and BH were significantly positively correlated with RMR, as well as BMI did, confirming the important role of BW and BH in modulating the basal metabolic rate, as these variables were already taken into account in early studies for the estimation of RMR.

WC, MAC and MAMC were found to be significantly positively associated with RMR. WC is a strong predictor for visceral adipose tissue, and it is currently and universally accepted as an independent cardiovascular risk marker for obese people[16]. Just few studies exploring and assessing the relationship between WC and RMR exist[17,18]. The correlation of MAC and MAMC with RMR could be explained by a higher accumulation of FM in the mid-upper arm in obese subjects, which is proportional to the accumulation of adipose tissue in other parts of the body.

BW as a significant predictor of increased RMR, and the higher percentage of FM and FFM in obese subjects, may lead also to a higher MAC and MAMC, hence to a higher RMR.

Several studies reported that RMR decreases paralleling aging, because of the loss of FFM and the increase in FM, since body mass and BC are the major predictors of EE[1]. In the present study no significant correlation was found among FM, FFM, BCM, MM, BCMI and RMR. FM was expected to contribute slightly to RMR, thus no significant correlation was obtained, and it is supported by previous evidence[19]. The influence of FM on RMR has not been univocally assessed, results being inconsistent across studies, even if it seems to play only a minor additional role.

FFM alone is well known to be related to RMR[20] though in the present study no correlation emerged, nor with values obtained by anthropometry neither by BIA, whereas a positive association was found between RMR and TBW. Special considerations should be highlighted when body composition of obese subjects is determined by BIA. In obese and overweight individuals the higher hydration of FFM is responsible for an overestimation of FFM and underestimation of FM. Additionally, the body build of obese subjects, especially abdominal obesity, concurs to the mentioned impairment of the results provided by the BIA[21]. Surprisingly in the present study TBW was significantly positively correlated with RMR, while FFM and FM were not. Even if BIA could not be completely reliable in our sample of obese participants, an increased total body water content mirroring a higher FFM may explain the positive association with RMR, that is notably higher as FFM increases.

Hence, the finding of a positive association between RMR and TBW, in absence of the same relation linking RMR and FFM, could be likely justified by the limited accuracy of BIA for FFM and FM assessment in such an altered hydration status that is obesity.

Otherwise, we evaluated FFM as a unique compartment, while growing evidence recognizes that FFM is energetically heterogeneous because it encompasses organs and tissues having a different metabolic rate[22].

Age did not show any correlation with RMR. Basal metabolism was shown to drop by 1%-2% per decade over the age of 20 to 75 years[23], and that aging process is accompanied by the apparent replacement of a certain proportion of MM by a gain in FM[24]. Actually, a potential reason for the inconsistency of our findings may be that mean age of our participants was lower than age of subjects in prior studies reporting an association between age and RMR[25].

Evaluating physical characteristics and the environmental conditions of the examination room, significant results were obtained only regarding BT. No significant results were obtained by the correlation analysis of RMR with BP, HR, SpO_2, MET, room humidity and atmospheric air pressure.

No significant correlation with RMR was found for the selected blood parameters, save for serum T_4 levels. The other blood parameters did not show any significant effect on the RMR, but it is likely that a detection of significantly abnormal values was difficult, since most subjects were on pharmacological therapy to maintain blood values in the normal range.

A wide spectrum of evidence supports the involvement of thyroid hormone in mechanisms responsible for thermogenesis, affecting EE and basal metabolic rate[26].

Resting energy expenditure (REE) has been shown to be very sensitive to modulation by thyroid hormone[27].

In the present study higher T_4 concentrations were associated with higher RMR. Although thyroid hormone concentration was within the normal range, a significant effect of T_4 concentration on RMR was however detectable. 19 subjects in the present study had hypothyroidism with no significant effect on metabolism, likely justified by levothyroxine chronic supplementation.

The effect of smoking status on EE has been at the centre of a number of studies attempting to assess the relationship linking the smoking attitude or its cessation to BW fluctuations[28,29]. The acute or short- term effect of cigarette smoking or nicotine leads to changes in metabolism that imply an increase in total EE or in RMR in few studies[30,31], despite this hypothesis remains to be confirmed. Moreover, smoking effect has been found to be different in obese smokers when compared to lean smokers[32]. In our study, smokers were all obese, and their RMR was increased by 10.4% than non-smokers' RMR. Mechanisms other than the direct influence on energy and metabolism may account for these results, as nicotine can affect appetite and sympathetic nervous system action.

An aspect that strengthens our finding is that the positive correlation between smoking and RMR exists even after patients refrained from smoking at least 2 h before the examination, eliminating the very short-term nicotine effects.

Disorders and diseases as well as medicines did not show any significant correlation with RMR, save OSAS, IGT, and impaired glycaemic status (including IGT, T2DM, and hyperinsulinism), that were significantly correlated with RMR.

In agreement with prior studies, we found that RMR was increased in OSAS obese subjects when compared to obese women without OSAS[33,34].

OSAS patients are usually obese, and abnormalities in intrathoracic pressure lead to an increased work of breathing, as well as frequent arousals and increased sympathetic activity, that may account for the increased EE[33,34].

Additionally, we found that RMR was significantly different in obese female subjects with an impaired glycemic status, when compared to their normoglycemic counterparts.

We considered subgroups including patients with IGT, diabetes mellitus, and hyperinsulinism, respectively, and subgroup analysis showed that RMR was significantly increased in patients with IGT or in whom with hyperinsulinism, whereas only a trend toward higher values of RMR was observed in patients with diabetes mellitus; previous studies had demonstrated an increased RMR in diabetic patients, and several mechanisms were implicated to explain the increased EE, such as the activation of energy- consuming metabolic processes- gluconeogenesis and other substrate cycles- and an increased sympathetic nervous system activity. We have to stress that in the subgroup of type 2 diabetic patients, those who were on metformin therapy were not considered separately, and it may account for the slight increase in RMR in the whole subgroup. Moreover, no significant difference has emerged from the comparison of RMR between diabetic subjects treated with metformin and non-treated patients. Metformin is responsible for improved glycemic control, thus it may avoid overcoming metabolic adaptations of RMR that typically occur in T2DM[35]. Therefore, the significant association between increased RMR and IGT and the tendency toward a higher RMR in type 2 diabetic patients could be due to the common soil of insulin resistance underlying the different aspects of glucose abnormalities; on the other hand, just a little body of evidence is available at present investigating the relationship between insulin resistance and RMR[36].

Definitive conclusions may not be drawn. First, sample size was relatively small. Moreover, we studied a group of white female subjects exclusively, and influence of both gender and ethnicity on RMR has been widely recognized.

It is noteworthy that 33 of the 77 subjects, originally entering the study, failed to achieve the steady state criterion for IC measurements, hence they were subsequently excluded.

In stable, spontaneously breathing patients, like those evaluated in the present study, anxiety or hyperventilation have been frequently addressed to impact the achievement of the steady state[37]. The failure in satisfying the steady state criterion could be attributable to the assumption of a very stringent definition of steady state: less than 10% for changes in V_{O_2} and V_{CO_2} over a period of 5 min. In existing literature less rigorous steady state criteria were applied, even if a weaker strength of correlation measurements with the 24-h REE was obtained when establishing a Vo2/Vco2 change by $\leqslant$ 15% or 20%. We adopted a more stringent definition of steady state in order to confer a higher accuracy to the results. We do not know if obesity may represent a limitation for the satisfaction of a very strict steady state criterion, and similar data are lacking for obese population at present.

In conclusion, the association among several anthropometric, physical and environmental factors, as well as disorders, diseases, medications, smoking habit and RMR was explored. Although significant results emerged from the correlation analysis, showing that RMR was positively related to BW, BH, BMI, WC, MAC, MAMC, TBW, BT, serum T_4 levels, levothyroxine and smoking, the reason for the significance was not always thoroughly clear, and it could be related to coincidental association within and between parameters, considering that RMR can range widely within a group of people.

The precise identification of factors influencing the RMR is not only an experimental concern, but it represents a real clinical challenge. IC is an important method allowing an individualized obese patient care. Defining factors interfering with RMR will be useful to maximize the beneficial effects deriving from a tailored therapeutic approach. Moreover it would prompt further research in order to address and to improve obesity management.

COMMENTS

Background

Obesity is spreading worldwide in an epidemic fashion. Research should be prompted to increase knowledge about mechanisms underlying metabolism regulation in order to optimize tools and potential care interventions.

Research frontiers

Currently technology offers tools that could be useful in a personalized approach to the management of obesity. Indirect calorimetry (IC) could be a technologic resource to improve the tailored treatment of obese subjects.

Innovations and breakthroughs

Because of the larger life expectancy and the presence of comorbidities linked both to the ageing process and to obesity itself, a variety of factors potentially affecting the resting metabolic rate (RMR) should be taken into account when considering obese subjects than their lean and healthy counterparts.

Applications

By understanding how resting energy expenditure (REE) is modulated in obese subjects, this study may represent a future strategy addressed to a technologically supported treatment and a more precise dietary intervention in this subset of patients.

Terminology

IC is a common method to assess exactly the REE, based on respiratory gas exchange, allowing defining thoroughly the characteristics of nutritional interventions.

Peer review

The authors examined selected factors potentially influencing the RMR in obese women. REE was evaluated using IC. The results suggest that this experimental approach could represent an interesting method for an individualized obese patient care.

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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